University of Alberta

BIOCHEMICAL, FUNCTIONAL AND MUTATIONAL ANALYSIS OF M-T2, THE TUMOR NECROSIS FACTOR RECEPTOR HOMOLOG OF MYXOMA VIRUS

by

Martha Patricia Schreiber ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

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University of Alberta
Edmonton

Department of Biochemistry

Canada T6G 2H7

474 Medical Sciences Building, Telephone (403)
Fax (403) 492-0886

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University of Alberta
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Sincerely,

Grant McFadden
Professor of Biochemistry

University of Alberta
Edmonton
Canada T6G 2H7

Department of Biochemistry
474 Medical Sciences Building, Telephone (403)
Fax (403) 492-0886

July 2, 1996

Faculty of Graduate Studies and Research
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University of Alberta
Edmonton, Alberta

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Sincerely,

Krishna Rajarathnam

I wish to know God's thoughts...
...the rest are details.

Albert Einstein

God has made everything beautiful in its time. He has also set eternity in the hearts of all; yet they cannot fathom what God has done from beginning to end.

Ecclesiastes 3:11

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M Schreiber

July 23, 1996

Martha Schreiber
17 Quinpool Crescent
Nepean, Ontario
K2H 6H9

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Biochemical, Functional and Mutational Analysis of M-T2, the Tumor Necrosis Factor Receptor Homolog of Myxoma Virus submitted by Martha Patricia Schreiber in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Grant McFadden (Supervisor)

Dr. Chris Bleackley

Dr. David Brindley

Dr. Larry Guilbert

Dr. Carl Ware (External Examiner)

Date: July 19, 1996

To my parents

Bertus and Alice Schreiber

ABSTRACT

The myxoma virus M-T2 protein shares extensive amino acid sequence homology with the ligand binding domains of the cellular tumor necrosis factor (TNF) receptors (TNFRs), and is an important virulence factor for myxoma virus infection in rabbits. Myxoma T2 protein and rabbit, mouse, and human TNF were expressed independently from vaccinia virus vectors. Rabbit cells showed strict species specificity and were only sensitive to cytolysis by rabbit TNF. However, growth of M-T2 non-expressing myxoma and vaccinia viruses in rabbit TNF-sensitive cells was only modestly reduced in the presence of rabbit TNF, suggesting that poxviruses possess additional anti-TNF mechanisms. M-T2 protein and rabbit TNF were purified to apparent homogeneity, and M-T2 not only blocked the binding of radiolabeled TNF to rabbit cell surfaces, but was able to protect mouse and rabbit cells from lysis by rabbit TNF at molar ratios comparable with soluble versions of host TNFRs. M-T2 was secreted from infected cells as monomers and disulphide-linked dimers, both of which bound rabbit TNF with affinities comparable to the affinities of mammalian TNFs with their receptors. However, the dimeric M-T2 form was a far more potent TNF-inhibitor, presumably because it can more effectively prevent dimerization of TNFRs. M-T2 protein was continuously synthesized throughout myxoma infection, and was secreted as an N-linked glycoprotein. M-T2 mutational analysis revealed that the first three cysteine rich domains, which share homology with TNFRs, were essential for TNF binding and inhibition of lysis whereas the C-terminus of M-T2 was important for efficient secretion from infected cells. M-T2 also inhibited the cytolytic activity of a second ligand, distinct from TNF, expressed on the surface of RL-5 cells. Moreover, intracellular M-T2 expression prevented myxoma-infected RL-5 cells from undergoing apoptosis. Mutant myxoma viruses containing M-T2 C-terminal truncations were generated, and one M-T2 mutant, ΔL113, was incapable of binding rabbit TNF, but retained full ability to inhibit virus-induced apoptosis of RL-5 cells.

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LIST OF ABBREVIATIONS

araC	arabinosyl cytosine
bp	base pair
BSA	bovine serum albumin
CD40L	CD40 ligand
Con A	concavalin A
CRD	cysteine rich domain
crm	cytokine response modifier
CTL	cytotoxic T lymphocyte
ds	double stranded
DMEM	Dulbecco's modification of Eagle's medium
EEV	extracellular enveloped virus
EGF	epidermal growth factor
eIF-2α	eukaryotic initiation factor 2α
FasL	Fas ligand
FBS	fetal bovine serum
FITC-12-dUTP	fluorescein-12-dUTP
HRP	horseradish peroxidase
HSV	herpes simplex virus
ICE	interleukin-1β converting enzyme
IFN	interferon
IL	interleukin
IMDM	Iscove's modification of Eagle's medium
INV	intracellular naked virus
kbp	kilobase pair
LPS	lipopolysaccharide

LT	lymphotoxin
m.o.i.	multiplicity of infection
MRV	malignanat rabbit fibroma virus
NCS	newborn calf serum
NK cell	natural killer cell
orf	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pcr	polymerase chain reaction
p.f.u.	plaque forming unit
PKR	double stranded RNA dependent protein kinase
PLA_2	phospholipase A_2
RCA	*Ricinus communis* agglutinin
SDS	sodium dodecyl sulphate
SFV	Shope fibroma virus
SLP	synthetic late promoter
SPI	serine protease inhibitor
TdT	terminal deoxynucleotidyl transferase
TGF-α	transforming growth factor α
TIR	terminal inverted repeat
tk	thymidine kinase
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
VGF	vaccinia growth factor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

LIST OF RECOMBINANT VIRUSES

vMyxlac	control myxoma virus (strain Lausanne) with wild-type phenotype; (*LacZ*$^{+}$);parental virus for vMyxt2g and vMyxΔC-terminus
vMyxt2g	myxoma T2 "knockout" virus: myxoma virus in which both copies of M-T2 were inactivated by insertional mutagenesis' (*LacZ*$^{+}$)
vMyxt2r	myxoma T2 reconstituted virus: myxoma virus in which both copies of M-T2, inactivated in vMyxt2g, have been reconstituted; (*LacZ*$^{-}$)
VV-601	control vaccinia virus (strain WR), with *LacZ* inserted into the tk locus
VV-MT2	vaccinia virus (strain WR) engineered to over-express M-T2; (*LacZ*$^{+}$)
VV-RaTNF	vaccinia virus (strain WR) engineered to express rabbit TNFα; (*LacZ*$^{+}$)
VV-MuTNF	vaccinia virus (strain WR) engineered to express mouse TNFα; (*LacZ*$^{+}$)
VV-HuTNF	vaccinia virus (strain WR) engineered to express human TNFα; (*LacZ*$^{+}$)
VV-T2RaTNF	vaccinia virus (strain WR) engineered to over-express and secrete rabbit TNFα, and contains M-T2 leader sequence/coding sequence of mature rabbit TNFα (*LacZ*$^{+}$)

Viruses Expressing M-T2 C-Terminal Truncations

Vaccinia Virus	**Myxoma Virus**
VV-MT2-ΔL113	vMyx-MT2-ΔL113
VV-MT2-ΔN169	vMyx-MT2-ΔN169
VV-MT2-ΔE174	vMyx-MT2-ΔE174
VV-MT2-ΔY236	vMyx-MT2-ΔY236
VV-MT2-ΔI249	vMyx-MT2-ΔI249
VV-MT2-ΔI260	vMyx-MT2-ΔI260
VV-MT2-ΔI279	vMyx-MT2-ΔI279
VV-MT2-ΔD303	vMyx-MT2-ΔD303

Vaccinia Viruses Expressing M-T2 Deletions of the Cysteine-Rich Domains

VV-MT2ΔCRD1
VV-MT2ΔCRD2
VV-MT2ΔCRD3

CHAPTER I - GENERAL INTRODUCTION

Throughout recorded history, poxviruses have been the causative agents of various human infectious diseases and the study of poxviruses has resulted in significant contributions to science and medicine (1). For centuries, devastating pandemics of smallpox claimed millions of lives, but in 1798 Edward Jenner's demonstration that previous exposure to cowpox could protect against smallpox infection led to a method of prevention against epidemics of the dreaded scourge. By 1977, smallpox had been globally eradicated through a major international effort organized by the World Health Organization to immunize populations worldwide. As the largest of the eukaryotic viruses, poxviruses were the first to be visualized microscopically, grown in tissue culture, physically purified and chemically analyzed. Moreover, the study of gene expression in vaccinia virus was instrumental in clarifying the structure of eukaryotic mRNA (1). Although poxviruses are no longer categorized as major human pathogens, recent studies on poxvirus-host immune interactions have contributed to our understanding of strategies that eukaryotic viruses use to evade host immune and inflammatory responses, and mechanisms that higher order vertebrates use to combat infection (2, 3). Recombinant poxviruses are presently being engineered to express antigens from a wide variety of animal and human pathogens in the hopes of using them as potential vaccine vectors (4). Furthermore, the recent identification of poxviral homologs of various cellular receptors, growth factors and effectors of signal transduction cascades has led to the poxviral genomes being described as "Brazilian rain forests", encoding for proteins with potential therapeutic value as pharmaceutical agents (5, 6).

POXVIRUS BIOLOGY

Classification and Physical Properties of Poxviruses

Poxviruses are a family of eukaryotic viruses characterized by a large complex virion containing enzymes involved with mRNA synthesis, a single linear double-standed (ds) DNA genome 130-300 kilobase pairs (kbp) in length with covalently closed hairpin termini and terminal inverted repeats (TIRs), and the ability to replicate within the cytoplasm of infected cells (1, 7). The virion is large, 200 to 400 nm long, and appears oval or brick-shaped with a biconcave nucleoprotein core and two lateral bodies surrounded by a lipoprotein bilayer membrane, and contains more than 100 polypeptides. Poxviruses exist in two morphologically distinct infectious forms, termed intracellular naked virus (INV) and extracellular enveloped virus (EEV). INV, which remains within the cytoplasm, comprises the majority of the infectious progeny whereas EEV, which is released from the infected cell, acquires an additional lipid envelope. EEV is important for the efficient viral dissemination *in vivo* and *in vitro*, and protection against live virus challenge requires antibody to EEV proteins (1, 7). In addition to encoding structural proteins and factors involved in transcription and DNA replication, the poxviral genome is large enough to encode factors which modulate the host immune response to infection. Genes essential for virus structure and replication that are highly conserved amongst poxviruses are clustered within the central region of the genome, whereas genes encoding virulence factors that are dispensible for growth in tissue culture are enriched within the terminal inverted repeats (8, 9). Poxviruses are divided into two subfamilies: the Chordopoxviridae (vertebrate poxviruses) and the Entemopoxviridae (insect poxviruses) (1). The Chordopoxviridae are further subdivided into eight genera on the basis of antigenic similarity, host range, and genomic cross-hybridization, (summarized in Table I-1) which include the Orthopoxviruses, Parapoxviruses, Avipoxviruses, Capripoxviruses, Leporipoxviruses, and Suipoxviruses.

Replication Cycle of Poxviruses

The poxviral replication cycle has been studied extensively, using vaccinia virus as a prototype, and consists of virus entry and uncoating, regulated gene expression, DNA replication, virion assembly, maturation and release (1, 9). The entire life cycle of poxviruses takes place within the cytoplasm of the infected cell, independent from the nucleus as poxviruses are unique in that they encode virtually all of the enzymes and factors required for mRNA synthesis and replication (9). Little is known about the mechanism of virus entry, although enveloped viruses in general enter cells either by fusion with the plasma membrane or by a low pH-dependent endosomal route (1, 7). Once naked cores are released into the cytoplasm, transcription is initiated and functional capped and polyadenylated mRNA are produced within minutes. Gene expression is temporally-regulated with three classes of genes, early, intermediate and late, transcribed in a strictly regulated cascade (1, 9-11). For vaccinia virus, transcription of early mRNAs begins immediately and continues for several hours before ceasing. The newly synthesized protein products are required for a secondary uncoating, which releases the viral genome and makes it accessible for DNA replication. Replication occurs within cytoplasmic factories and DNA concatemers are produced which are subsequently resolved into unit-length monomers. Intermediate gene expression requires both *trans*-acting early gene products and DNA templates that have undergone *cis* replication which presumably frees the template of putative repressors. Intermediate gene products then drive the transition to late gene expression. Virus assembly and maturation is a complex process that involves extensive wrapping with cellular membranes (1, 7). Some of the INV then acquires a double layer of Golgi-derived membrane before being released from the cell as EEV.

Orthopoxviruses

The Orthopoxviruses include variola, the causative agent of smallpox, vaccinia, the virus used as the smallpox vaccine, and cowpox, which is thought to have been the

Jennerian vaccine (12-14). For centuries, cowpox was recognized as a bovine disease. However, the cow may only be an accidental host of the virus, and currently it is thought that cows, cats and even humans can become infected from a rodent reservoir host. Over the last 50-200 years, vaccinia virus has appeared as a distinct species of Orthopoxvirus, different from cowpox virus. The origin of vaccinia virus is unknown, although it has been postulated that vaccinia virus was derived from variola virus, cowpox virus or an extinct virus by serial passage in cow or human skin. Alternatively, vaccinia virus may represent a hybrid between cowpox and variola viruses (12, 13). Because its origins are unknown, there is no obvious candidate for animal studies on vaccinia virus. Instead intranasal or intracranial innoculations of mice have been used to study vaccinia virus virulence (15-18).

Both the vaccinia and variola genomes have been sequenced and are organized with essential genes encoding structural elements, enzymes required for transcription and replication factors mapping toward the center, and non-essential genes encoding host range and virulence determinants mapping toward the termini (19-23). The vaccinia and variola virus genomes are 191 and 186 kbp in length, respectively, and encode approximately 200 genes.

Leporipoxviruses

Shope Fibroma Virus

The Leporipoxviruses are restricted to rabbits, squirrels and hares. Shope fibroma virus (SFV) was first isolated in 1932 from a subcutaneous lesion on a wild cottontail rabbit (reviewed in 24, 25). It infects the wild cottontail rabbit (*Sylvilagus floridanus*) as well as rabbits of other genera including the European domestic rabbit (*Oryctolagus cuniculus*), and transmission in the wild occurs largely via dermal innoculation by biting arthropod vectors. Viral replication is restricted to dermal and subcutaneous sites and infection rarely causes disease symptoms in immunocompetent rabbits except for a localized benign cutaneous fibroma. For SFV infection of *Sylvilagus* rabbits, lesions can persist for many months before regressing, whereas in *Oryctolagus* rabbits, recovery is usually complete within several weeks.

The SFV genome is 160 kbp and has been cloned into bacterial plasmid vectors (26, 27). In addition, the sequence is known for the 12.4 kpb TIRS (28).

Myxoma Virus

Myxoma virus is the causative agent of myxomatosis in rabbits, although the disease profile of myxoma infection is greatly influenced by the genetic background of both the virus strain and the rabbit host. The virus was originally discovered at the end of the 19th century in Uruguay, when imported European rabbits (*Oryctolagus cuniculus*) were suddently stricken with an unknown illness. In its natural hosts, the North American brush rabbit and the South American tropical forest rabbit (*S. bachmani* and *S. brasiliensis*, respectively), myxoma virus causes only a mild infection with minor symptoms (3, 24, 25, 29, 30). In *Oryctolagus*, the disease caused by the prototypic Lausanne strain (S. American form) is characterized by the appearance of large, raised dermal lesions at the primary site of infection, followed by dissemination of the virus through the lymphoreticular system, and the appearance of multiple internal and external lesions at secondary sites particularily the nose, eyes and ears by 6-7 days (3, 6, 24, 25, 29, 31). The cutaneous lesions often become congested and necrotic. Infection results in generalized immune dysfunction, and supervening Gram negative bacterial infections of the respiratory tract and conjuctiva are often observed and contribute to the lethality of the disease. Mortality can be >99% in *Oryctolagus*, and death occurs within 1-2 weeks. In contrast to the S. American form, the disease caused by the California strain of myxoma in *Oryctolagus* rabbits, is characterized by only minor external symptons such as inflammation and edema of the eyelids; however, rabbits succumb within one week, and convulsions often precede death. Unlike SFV, myxoma virus is able to replicate in lymphocytes and monocytes and viral antigens can be found throughout the lymphoreticular system during infection, leading to the hypothesis that the ability to replicate within lymphoid cells may be a determinant of the capacity of the virus to disseminate within the infected animal (24, 31, 32)

In the early 1950's, myxoma virus was deliberately released in Southern Australia in at attempt to control feral European rabbits populations. Rabbits are not native to Australia, but instead had been imported in the last century by settlers and, with no natural predators, they soon overtook large areas of the country. Although the inital myxoma release decimated rabbit numbers, resistant rabbits repopulated the area and the dominant myxoma field strains became progressively attenuated (29, 33). Although myxoma is now endemic in the country, Australia's rabbit population presently approaches the level found before the virus was first released. The release of myxoma virus in Australia provided a unique opportunity to study the evolution of a virus within a wild population. Field strains have been isolated which vary in pathogenicity and the dominant field strains today are those of intermediate virulence (29, 34).

The myxoma genome is approximately the same size as SFV, 163.6 kbp in length, and the two viruses show considerable cross hybridization under conditions of moderate stringency, indicating a close evolutionary relationship (24, 35). Infection with SFV renders rabbits resistant to subsequent challenge with myxoma virus. Detailed restriction maps have been constructed for myxoma and the genome cloned into bacterial plasmid vectors (35, 36). Figure I-1 outlines the Bam HI map of the myxoma genome, indicating the locations of the various virulence factors that have been characterized.

Malignant rabbit fibroma virus (MRV) is a natural recombinant between SFV and myxoma, and establishes a highly virulent generalized infection in European rabbits similar to myxomatosis (31, 36). Sequencing studies have shown that the MRV genome consists mainly of myxoma virus, with 7978 base pairs (bp) of corresponding SFV DNA replacing the left TIR and adjacent sequence, and 4674 bp replacing the related sequence at the right end of the genome (37).

Myxoma virus provides an excellent model for examining the molecular mechanisms of poxvirus pathogenesis. Myxomatosis has a well defined and predictable pathology in *Oryctolagus*. Moreover, the history of the virus has been described and its natural host is known. Recent examination of myxoma virus at the molecular level has led to the discovery of various viral homologs of cellular growth factors and cytokine receptors which contribute to the virulence of the virus (3, 6, 30).

POXVIRAL PROTEINS IMPLICATED IN VIRAL PATHOGENESIS

Poxviruses have been found to encode for a number of proteins which alter the host immune response to infection and which function either intracellularly or extracellularly to affect the virulence of the virus (2, 3, 6, 7, 30, 38-40). The term virokine was proposed to designate viral proteins which mimic host ligands or related soluble immune regulators, following the discovery that homologs of host growth factors and complement inhibitors were encoded by vaccinia virus (41, 42). Later, the term viroceptor was used to describe viral homologs of cellular receptors which act to bind and sequester host ligands and prevent their interaction with their cognate receptors (43). Cytokine response modifier (crm) is a more general term that refers to a viral protein which modulates host cytokine induction, secretion, activation, or ligand function (44). It is likely that viruses originally acquired many of these genes from the host cells they infect and have subverted their function in order to confer a selective advantage to the virus (5). The different virokines, viroceptors and cytokine response modifiers encoded by poxviruses are summarized in Table I-2.

Virokines

Inhibition of the Complement Cascade

The complement system is the primary humoral defense mechanism against infection. It can be activated by either the classical pathway which requires a specific antibody-antigen interaction or the alternative pathway independent of antibody by certain molecules such as lipopolysaccharide (LPS), and consists of a cascade of enzymatic reactions which culminate in the generation of peptides that initiate inflammation and the formation of a membrane attack complex that damages the surface membrane of infected cells or enveloped viruses (45). Various microorganisms have devised strategies to evade the complement system, including vaccinia virus, Herpes simplex virus (HSV) types I and II, Epstein-Barr virus, *Trypanosoma cruzi*, *Candida albicans* and *Plasmodium falciparum* (46). Vaccinia virus encodes a secreted 35 kDa protein (gene *C21L*) that shares sequence similarity with C4b-binding protein, and binds to C3b and C4b, thus blocking the activation of both the alternative and classical pathways (42, 47, 48). Both vaccinia and variola encode similar putative complement receptors, B5R and B6R respectively, which are present on the surface of infected cells and extracellular virus particles, and although the ability of these proteins to bind complement factors are unknown, they have been proposed to protect extracellular virus or infected cells from complement or to allow virus particles to bind to cells bearing complement receptors (49-51). The vaccinia B5R protein is required for the formation of EEV, influences plaque size and host range, and contributes to viral virulence in mice (49, 50, 52). A gene homologous to *B5R*, *ps/hr*, is encoded by rabbitpox virus which also influences plaque size and host range (53).

Poxviral Homologs of Growth Factors

Various poxviruses, including variola, vaccinia, myxoma and SFV, encode homologs of epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) which contribute to viral virulence (54). Vaccinia growth factor (VGF), the prototypical poxviral growth factor, is a secreted 19 kDa protein that shares significant sequence similarity to EGF and TGF-α, including six cysteine residues important for folding and biological activity. VGF is thought to stimulate the growth of uninfected cells in order to enhance subsequent virus replication (41, 55, 56). Similar to host EGF and TGF-α, VGF is first synthesized as a transmembrane glycoprotein that is subsequently cleaved for secretion, and VGF competes with EGF for receptor binding, stimulates receptor autophosphorylation and is mitogenic for certain cells (57-59). Although VGF is not required for growth in tissue culture, deletion of the VGF gene results in virus attentuation in a mouse pathogenesis model (60). Both recombinant MRV and myxoma viruses containing deletions within their growth factor genes were significantly attentuated in rabbits, indicating that these growth factors are important virulence factors (32, 61). Moreover, TGF-α, VGF and Shope fibroma growth factor could all replace myxoma growth factor (MGF) in myxoma virus without phenotypic alterations in rabbits (62).

The *A2R* gene of the Parapoxvirus, orf virus, shows amino acid sequence homology to the mammalian vascular endothelial growth factor (VEGF), an important regulator of angiogenesis, and infection of sheep with orf virus results in extensive capillary proliferation and swelling of the dermis (63) .

Viroceptors

Tumor Necrosis Factor Receptor Homologs

The first example of a virally encoded homolog of a cellular cytokine receptor was the T2 gene of SFV (64). In 1987, the SFV T2 (S-T2) open reading frame (orf), the second open reading frame from the termini, was sequenced and shown to be expressed as a virus early gene (28, 65). When the cellular type I and type II tumor necrosis factor (TNF) receptors (TNFR) were cloned two years later, it was realized that the *S-T2* gene belonged to the TNFR superfamily that now includes important immunomodulators such as Fas, CD40, CD30, CD27, OX-40 and 4-1 BB (40, 66). S-T2 was independently expressed from COS cells and shown to bind TNF and lymphotoxin α (LTα), the natural ligands for the TNFRs (67). A TNFR homolog, designated *M-T2*, was also found in myxoma virus within the terminal inverted repeats and was shown to be an important virulence determinant for myxoma. The majority of European rabbits infected with a recombinant myxoma virus in which both copies of *M-T2* were inactivated by insertional mutagenesis were able to mount an effective immune response and completely recover (43). The characterization of the M-T2 protein is the subject of this thesis.

TNFR homologs have also been found in other poxviruses, such as cowpox and variola (23, 68). The vaccinia virus genome contains two discontinuous and presumably nonfunctional fragments of TNFR genes, but this virus is highly attentuated by passage outside a true vertebrate host (19, 20). Figure I-2 summarizes homologies between the different poxviral and cellular TNFR family members, showing conserved cysteines present within the cysteine rich domains (CRDs).

Interferon-Receptor Homologs

Although initially discovered because of their antiviral activity, the interferons (IFNs) are also recognized as important modulators of the immune system and effect cell growth and differentiation. There are three types of IFN: IFN-α (leukocyte), IFN-β (fibroblast) and IFN-γ (immune) (69-72). IFN-α and IFN-β share a common receptor and are termed type I IFNs, whereas IFN-γ, also called type II IFN, has a separate receptor. Expression of type I IFNs, which enhance the ability of cells to resist viral infection, is induced by viral infection of virtually all somatic cells, whereas IFN-γ is produced predominantly by activated T-lymphocytes and natural killer (NK) cells. The importance of IFN in the resolution of poxviral infections has been well documented (14, 40, 73). Infection of immunodeficient mice with a recombinant vaccinia virus expressing murine IFN-γ resulted in a dramatic attentuation in pathogenicity (74), whereas treatment of vaccinia virus-infected mice with a monoclonal antibody against IFN-γ enhanced the pathogenicity (75).

The major secreted protein from myxoma virus, M-T7, shows sequence similarity to the ligand binding domain of the cellular IFN-γ receptor (IFN-γR), binds and inhibits the activity of rabbit IFNγ, and is an important virulence determinant for myxoma virus (76-78). IFN-γR homologs have also been identified in SFV, vaccinia, variola, ectromelia and swine pox viruses (19, 22, 23, 79-81), and IFN-γ binding proteins have been detected in supernatants of cells infected with rabbitpox, cowpox and camelpox viruses (81, 82). Although the myxoma IFN-γR homolog is similar to cellular IFN-γR in that it shows species specificity by binding and inhibiting only rabbit IFN-γ (77), the ectromelia, vaccinia, cowpox and camelpox IFN-γ homolgs display broader specificity and bind and inhibit IFN-γ from a number of different species (81, 82).

Vaccinia virus has also been shown to encode for a soluble type I interferon receptor, *B18R* (83). However, B18R, unlike cellular type I IFN receptors, is a member of the immunoglobulin superfamily and is related to the interleukin-1 (IL-1) receptors. B18R is secreted from infected cells, binds human IFN-α with high affinity, and unlike cellular type I IFN receptors, displays broad species specificity binding human, rabbit, bovine, rat and mouse type I IFNs. Other orthopoxviruses including cowpox, elephantpox and rabbitpox also appear to express an inhibitor of type I IFNs.

Interleukin-1β–Receptor Homologs

Vaccinia virus (present in WR but truncated in Copenhagen) and cowpox viruses encode soluble versions of the cellular IL-1 receptors, with homology between the viral proteins and cellular receptors limited to the external IL-1 binding domains (17, 18). IL-1β is known to synergize with TNF in the early inflammatory responses to viral infection (84). Although both the membrane bound IL-1 receptor and the natural IL-1 receptor antagonist protein bind IL-1α and β, the vaccinia virus (strain WR) homolog, B15R, only binds IL-1β. There is conflicting evidence about the contribution of the IL-1β receptor to virus virulence, depending whether mice were infected intranasally, or intracranially (17, 18). The B15R homolog in variola is fragmented (22, 23), and interestingly, vaccinia virus strains used for smallpox vaccination induced higher levels of postvaccinial encephalitis if they did not express B15R (18).

Interleukin-8 Receptor Homologs

Sequencing of the swinepox genome revealed a viral orf, *C3L*, displaying homology with the human gene encoding the low affinity human interleukin-8 (IL-8) receptor, but the protein product of this gene has not been characterized (80). Moreover, the *K2R* orf also shows homology to the low affinity IL-8 receptor, and may be able to bind chemokines, although it too has not been characterized (80).

Other Poxviral Cytokine Response Modifiers And Virulence Factors

Viral proteins can inhibit cytokine function by modulating the post-translational processing of cytokine precursors, and interfering with their proteolytic activation and/or intracellular transport. Poxviruses have been shown to encode serpins (serine protease inhibitors, SPI) which act as pseudosubstrates of serine proteinases, forming stable complexes with the proteinase and thereby inactivating it, and the various poxviral serpins have been shown to affect poxvirus pathogenesis (85) .

SPI-2 (crm A)

Recently, a viral serpin that acts as an inhibitor of the IL-1β converting enzyme (ICE) was identified in Orthopoxviruses (86). The SPI-2/crmA serpin was first described in cowpox, but homologs have been identified in other Orthopoxviruses including vaccinia, strain WR but appears only as interrupted ORFs in vaccinia strain Copenhagen, variola and rabbitpox (19, 20, 22, 23, 85-87). Excessive infiltration of inflammatory cells was observed in chicken chorioallantoic membranes lesions induced by cowpox virus bearing deletions of the *crmA* gene compared to lesions induced by wild-type virus, which can in part be accounted for by the ability of crmA to inhibit ICE, and thus impair the release of mature IL-1β (88). However, a SPI-2 mutant in vaccinia virus did not affect virulence in a murine model (89).

An ICE-like proteinase has been implicated in Fas-mediated apoptosis (90, 91), and the crmA protein has been shown to prevent programmed cell death and to block Fas- and TNF-induced apoptosis (92, 93). Thus, the *crmA* gene may also function to inhibit the apoptosis response in virus-infected cells, although the anti-apoptotic effect of crmA has only been studied in heterologous systems.

SPI-1 and SPI-3

The *SPI-1* gene, encoding a serpin with homology to *SPI-2/crmA*, was identified by sequencing near the right TIR of vaccinia virus (94, 95). SPI-1 homologs have been identified in cowpox, variola and rabbitpox, but the reactive center region is different from that of SPI-2 suggesting that SPI-1 inhibits a different serine proteinase (85). Inactivation of the *SPI-1* gene has no effect on pock color in chicken chorioallantoic membranes or on virus virulence in mice (96, 97), but rabbitpox virus with SPI-1 deletions is unable to plaque on some cell types, which suggests that SPI-1 may prevent apoptosis within certain otherwise non-permissive cells (85).

The Orthopoxviruses vaccinia, variola, rabbitpox, cowpox and raccoonpox, all encode a third serpin, SPI-3, which is non-essential for growth in tissue culture and does not affect the virulence of vaccinia, rabbitpox or cowpox viruses in mice, but does prevent cell-cell fusion following infection (85).

SERP-1/SPI-4

Myxoma virus encodes a gene with homology to serpin family members, designated *SERP-1* (or *SPI-4*), that is required for pathogenesis in European rabbits, but the corresponding orf is fragmented within the SFV genome (98). SERP-1 is expressed as a secreted glycoprotein that is able to bind and inhibit a variety of extracellular serine proteinases, including plasmin, urokinase, and tissue plasminogen activator, but the biologically relevant target in infected rabbit tissues has not yet been identified (98, 99). In rabbits infected with a SERP-1 inactivated virus, more effective infiltration of inflammatory leukocytes at the site of infection led to an attentuated myxomatosis (98). Moreover, the anti-inflammatory properties of SERP-1 were further demonstrated in a rabbit atherosclerosis model. When purified SERP-1 was infused into the arterial wall during balloon angioplasty, the subsequent atherosclerotic plaque development was inhibited (100). Although it is uncertain whether SERP-1 targets a member of the cytokine network, proinflammatory cytokines that require extracellular proteolytic activation have been suggested as targets (3, 6).

Additional Anti-Interferon Defenses

In addition to IFN-binding proteins, poxviruses use other strategies to evade a host IFN-mediated antiviral response. Two antiviral pathways have been well characterized for IFN, induction of dsRNA dependent protein kinase (PKR), and induction of 2'-5'-oligoadenylate synthetase. In the presence of dsRNA, PKR catalyzes the phosphorylation of eukaryotic Initiation Factor 2α (eIF-2α) which leads to an inhibition of protein synthesis by irreversible binding of eIF-2α with the guanine nucleotide recycling factor that is required for continued initation of translation. The enzyme 2'-5'-oligoadenylate synthetase is activated by dsRNA and catalyses the formation of 2'-5'-linked oligoadenylates, which in turn activate a latent ribonuclease, RNase L. Degradation of mRNA and rRNA leads to a general block in translation (69, 70, 72). Vaccinia virus expresses a dsRNA-binding protein from the *E3L* gene that displays homology to PKR, binds dsRNA and competitevely inhibits PKR activation, and perhaps also 2-5-oligoadenylate synthetase (101, 102). Moreover, vaccinia virus K3L protein shares sequence homology with the N-terminus of eIF-2α, functions as a substrate for PKR to prevent the phosphorylation of eIF-2α, and abrogates the IFN-mediated inhibition of viral replication in tissue culture (103). A *K3L* homolog is present in variola (21).

Host Range Genes and Inhibitors of Apoptosis

Very recently, a myxoma virus gene product, M-T5, was shown to prevent complete shut-off of protein synthesis and apoptosis in infected rabbit T-lymphocytes (104). A T5-inactivated myxoma virus was extremely attentuated in European rabbits: infected rabbits did not display any of the characteristic features of myxomatosis and the virus did not spread to secondary sites. M-T5 expression allows productive infection of immune cells such as peripheral lymphocytes, facilitating the dissemination of the virus within the infected rabbit.

The M-T5 gene product appears to act in a similar fashion to the cowpox virus CHOhr gene product. CHO cells undergo shutoff of protein synthesis and rapid apoptosis when infected with vaccinia virus. However, when CHO cells were infected with vaccinia virus expressing the cowpox *CHOhr* gene, viral late genes were expressed and there was a delay in infection-induced apoptosis (105). The vaccinia *E3L* gene, encoding the PKR homolog, is also required to prevent apoptosis and allow productive infection within fibroblasts (106). PKR itself can induce apoptosis in certain cell types, and the E3L protein may block multiple PKR antiviral effects.

Moreover, two other myxoma-encoded proteins, M-T2 and M11L, have been shown to be important in inhibiting apoptosis and facilitating productive infection in a rabbit $CD4^+$ T cell line (107). The anti-apoptotic effect of M-T2 is further characterized in this thesis. M11L was originally characterized as an important virulence factor for myxoma virus. When European rabbits were infected with myxoma virus containing a disrupted M11L gene, benign fibroma-like lesions containing massive influxes of infiltrating leukocytes were observed and the rabbits completely recovered from infection (32). M11L is expressed as a small transmembrane protein and may be an example of a cell surface viroceptor (108). However no target for M11L action has yet been identified.

Downregulation of MHC

Cytotoxic T lymphocytes recognize intracellular viral antigens in association with class I MHC molecules on cell surfaces and this recognition is an important cell-mediated host defense against infection (109). Infection with myxoma virus results in a loss of class I MHC molecules from the surface of infected cells, which may represent an important mechanism for infected cells to escape cell-mediated immune recognition (110). The viral protein(s) affecting this response has not been identified, although it appears to be a late gene product. Moreover, myxoma virus induces extensive CD4 downregulation and dissociation of $p56^{lck}$ from CD4 in a rabbit $CD4^+$ T cell line, although the mechanism for this effect has not been determined (111).

Synthesis of Steroid Hormones

The enzyme 3β-hydroxysteroid dehydrogenase, which participates in the synthesis of steroid hormones, was recently found to be encoded by vaccinia virus and is a virulence factor for infection of mice (112). Although it has not been determined *in vivo* whether steroids are actually synthesized *de novo*, the possibility exists that immunosuppressive glucocorticoids are being synthesized which reduce the efficiency of virus clearance by the host. Alternatively, any glucocorticoids synthesized could stimulate metabolism in neighboring cells causing them to proliferate, and leading to enhanced viral replication when they, in turn, become infected.

STRATEGIES OTHER VIRUSES USE TO INHIBIT APOPTOSIS

Viruses other than poxviruses have evolved strategies to combat host immune responses. The discussion here will be limited to viral evasion of apoptotic responses, and the various strategies viruses use to inhibit apoptosis, presently identified, are summarized in Table I-3.

Both apoptotic and necrotic death have been well characterized for cells (113-115). Necrotic death is pro-inflammatory because the cell swells and eventually lyses, releasing the cytoplasmic contents to the environment. In contrast, during apoptotic death, the cell shrinks and fragments into membrane-bound vesicles as the chromatin condenses and degrades. Apoptotic cells, and any intracellular virus particles, are rapidly ingested by phagocytic cells, and thus apoptotic death is a protective mechanism of the host against viral infection. On the other hand, necrotic death of virus-infected cells provides an efficient mechanism for dissemination of non-budding viruses (116). Many viruses that have adapted to the host's apoptotic response encode factors which either directly inhibit apoptotic effectors or interfere with the apoptotic signal within infected cells. African swine fever virus, a virus related to poxviruses, and the Epstein-Barr virus each encode viral homologs of Bcl-2, a cellular inhibitor of apoptosis (117, 118). Epstein-Barr virus also expresses a second protein, LMP-1, which upregulates cellular *bcl-2* expression (119). The herpes virus $\gamma_1$34.5 protein prevents complete shutoff of protein synthesis and subsequent triggering of programmed cell death in human neuronal cells (120). The hepatitis B virus protein, HBx, forms complexes with the cellular transcriptional transactivator, p53, and inhibits p53-mediated apoptosis (121). Moreover, adenovirus encodes four proteins which interfere with different aspects of the TNF signal transduction pathway (122, 123). Baculovirus encodes two anti-apoptotic factors, the *p35* gene and *iap*, inhibitor of apoptosis (124, 125). Recently, cellular homologs of the baculovirus iap protein have been identified which associate with the p75 TNF receptor (TNFR) signaling complex (126), suggesting that the iap protein may inhibit apoptosis via this pathway.

Adenovirus Evasion of TNF Anti-Viral Responses

Adenoviruses express a number of proteins that affect the sensitivity of infected cells to cytolysis by TNF (122, 123, 127, 128). The E1A protein induces sensitivity to cytolysis by TNF, whereas the EIB-19K protein and three proteins encoded by the E3 region protect against sensitivity to TNF induced by E1A expression or the inhibition of protein synthesis. Induction of apoptosis by E1A in rat cells requires a block in proliferation, and the regions of E1A protein essential for inducing apoptosis also map with the regions required for binding to the cellular proteins pRb and p300, both of which regulate the expression of cell-cycle dependent genes (129). The E1B-19K protein is a distant homolog of Bcl-2, and prevents TNF-induced cytolysis of human and rat cells, but not mouse cells, blocking p53-induced apoptosis by an unknown mechanism (128). A second viral protein, E1B-55K, binds to and inactivates p53 (130, 131). E1A alone is inefficient at transforming cells, however, E1A acting together with E1B-19K or E1B-55K readily transforms cells (128). E1A stimulates DNA synthesis and cell proliferation, by releasing cellular proteins from complexes with pRb and p300, and when this occurs under conditions in which the cell cycle is blocked by p53, the cellular response to this is apoptosis (128, 132, 133). E1B-19K and E1B-55K both inhibit p53-mediated apoptosis (128). E1A mutants with deletions of the pRb and p300 binding sites failed to induce sensitivity to TNF, suggesting that binding to either of these cell growth regulators is sufficient to induce TNF sensitivity (122), and E1B-19K mutants have revealed that the ability of E1B-19K to inhibit apoptosis coincides with its ability to transform cells and to inhibit TNF cytolysis, suggesting that E1B-19K acts at a common step in the pathway to cell death (134, 135).

The E3-14.7K protein, present in all serotypes in which the E3 region has been sequenced, is a hydrophobic, non-membrane protein that localizes to the nucleus and cytoplasm, and inhibits cytolysis by TNF (122). E3-14.7K is thought to block TNF-induced activation of phospholipase A_2 (PLA_2) and cytolysis, and thus also to inhibit the inflammatory response by preventing the synthesis of leukotrienes and prostaglandins (122, 128). Moreover, when mice were infected with recombinant vaccinia virus expressing both TNF and E3-14.7K protein, the 14.7K protein increased the virulence of vaccinia virus expressing TNF by counteracting the antiviral effects of TNF (136).

E3-10.4K and E3-14.5K form a complex on the plasma membrane and together can inhibit cytolysis by TNF and downregulate the EGF receptor (137). The mechanism of action of the E3-10.4K/14.5K complex is unknown, but may involve inhibition of PLA_2 and arachidonic acid release by a different mechanism than E3-14.7K (122, 128).

THE TUMOR NECROSIS FACTOR AND THE TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILIES

In the late 19th century, William B. Coley, a New York surgeon, had some success in treating cancer patients by injecting them with killed bacteria, which induced in patients many of the symptoms of bacterial infection, including fevers and chills, and for some patients, also resulted in tumor regression or disappearance (138-141). Results were inconsistent, however, and Coley's treatment was subsequently abandoned with the development of radiation therapy and chemotherapy. By the 1950's, it was realized that components of the outer wall of Gram-negative bacteria such as LPS and BCG (from *Bacillus Calmette-Guerin*) were capable of promoting hemorrhagic necrosis of tumors in animals, and in minute quantities would induce fever which had a protective effect against infection, but in greater amounts led to shock and death. However, *in vitro* studies showed that neither LPS nor BCG were capable of killing tumor cells directly, and by 1986, Coley's toxin, or TNF, had been purified from the serum of rabbits and mice, and from HL-60 cell supernatants (142-144). Soon afterwards cDNAs for the different TNFs were isolated, and the genomic TNF loci cloned (145-150).

In parallel studies in the 1980's, researchers were actively working to isolate and clone a factor referred to as cachectin, presumed responsible for the accelerated lipid and protein metabolism associated with the profound anorexia or cachexia often observed with chronic disease. Beutler and associates were able to purify mouse cachectin to homogeneity based on its ability to suppress lipoprotein lipase activity (151). When both TNF and cachectin were sequenced, it was realized that they were identical molecules (152).

The purification and cloning of lymphotoxin α (LTα), a lymphokine with cell lytic activity produced by activated lymphocytes, otherwise referred to as TNFβ, predated that of TNF (153, 154). When the sequences of TNF and LTα were compared, it was realized that they were related molecules. The TNF and LTα genes reside within the major histocompatibility complex (chromosome 6 in humans, chromosome 17 in mice) and are separated by only about 1.1 kbp (155).

The TNF and TNF Receptor Superfamilies

The past decade has seen the characterization of a new family of cytokines and immune modulators, the TNF superfamily, which includes TNF, LTα, LTβ, ligands for

Fas, CD40, CD30, CD27, 4-1BB, and OX40, and the recently identified TRAIL (66, 156-159). All of the ligands are type II transmembrane proteins, except for LTα which is secreted from cells. Proteolytic cleavage of membrane-bound TNF results in a soluble, 17 kDa active species. All members display sequence similarity with homology restricted to the C-terminal 150 amino acids which form the extracellular receptor binding domains, and it is thought that all the ligands in the family adopt a similar tertiary structure (66, 156). Both TNF and LTα are active as trimers, and their X-ray crystal structures have revealed that their receptor binding domains fold into "jelly roll" β-sandwich motifs (160-162). The TNF related ligands assemble into trimers, and that they activate their cognate receptors via crosslinking (66, 156, 157).

Receptors for the TNF family members define a parallel corresponding superfamily, the TNFR superfamily, and include the type I and type II TNFRs, LTβR, Fas, CD40, CD30, CD27, OX40, 4-1BB, the nerve growth factor receptor, as well as poxviral homologs (66, 156-158). Members of the TNFR superfamily are characterized by multiple, often four-fold repeats of a cysteine-rich domain (CRD), consisting of about 40 amino acids, within their N-terminal extracellular ligand binding regions. The average homology between family members within the CRDs is around 25%. Figure I-2 summarizes the homologies between the different poxviral and cellular members of the TNFR superfamily, showing conserved cysteines present within the CRDs. M-T2 shares the greatest similarity with the type II, p75 TNFR (43). Homology between family members is mainly limited to the extracellular regions, and the intracellular regions are rather small (46-221 amino acids) and do not appear to possess catalytic activities. However, the type I TNFR, CD40 and Fas also show sequence similarity in their cytoplasmic regions, as do CD27 and 4-1BB.

For most members of the TNFR superfamily, soluble forms of the receptor capable of binding ligand exist *in vivo*, released by proteolysis of the membrane bound receptor (reviewed in 163-165). These shed forms of the receptors act as cytokine antagonists, competing with the cellular receptors for ligand binding, and neutralizing the bioactivity of the cytokine. In addition, TNF is stabilized in complexes with soluble TNFRs under certain conditions, with the slow release of biologically active TNF from the complex acting to maintain physiological beneficial TNF concentrations.

The various functions of the TNF family ligands and their receptors are summarized in Table I-4.

TNF/LTα and TNFRs

Biological Properties of TNF

The biological activities of the TNF and LTα have been the subject of numerous recent reviews and books, and as the two ligands bind the same two cellular TNFRs, LTα mirrors many of the activities of TNF (139, 141, 156, 158, 166-170). Although initially thought to be produced by activated macrophages, it is now recognized that TNF is expressed on a wide range of hemopoietic and non-hemopoietic cells as a 26 kDa membrane-bound molecule, which is proteolytically cleaved to yield an active, 17 kDa soluble species. The membrane bound form, however, is capable of killing target cells by direct cell-cell contact, and provides a co-stimulatory signal for B cell activation (171, 172). LTα expression is limited to T and B cells, and forms a cell surface complex with LTβ (173).

The TNFs are pleiotropic, multipotent cytokines that mediate numerous biological processes including inflammation, tissue remodelling, immune responses to infection, cytotoxicity, endotoxic/septic shock and cachexia (139, 166, 167, 174, 175). The TNFs act on numerous tissues, including adipose tissue, muscle, liver, skin, gastrointestinal tract, central nervous system, adrenal glands, skin, bone, cartillage, vascular endothelium, and a variety of hematopoeitic cells including neutrophils, eosinophils, monocytes/macrophages, and lymphocytes (141, 174-176). TNF enhances the expression of class I MHC molecules, induces fibroblast growth, increases neutrophil adhesion, activates polymorphonuclear leukocytes, augments NK cell cytotoxicity and stimulates the proliferation of IL-2 dependent T cells and B cell differentiation.

In addition to activating an immune response, TNF can also be directly cytotoxic to various tumor cells and cells infected with certain viruses, including vesicular stomatis virus, HSV, and Newcastle disease virus, and thereby inhibits viral replication (127, 177-183). Moreover, TNF can induce either apoptotic and necrotic cell death (184, 185). Many cells become sensitive to lysis by TNF only after treatment with protein synthesis inhibitors, and TNF is known to induce the synthesis of proteins which protect against cytolysis (177, 179). By shutoff of host protein synthesis, many viruses may thus render infected cells sensitive to the cytolytic activities of TNF. The importance of TNF in an antiviral response is attested to by the fact that adenoviruses encode four proteins which interfere with the cytolytic properties of TNF (122, 123). Moreover, when athymic, nude or sublethally irradiated mice were infected with recombinant vaccinia viruses expressing TNF, local expression of the cytokine resulted in a dramatic attentuation of viral pathology, and clearance of the virus was associated with elevated neutrophil numbers within 24 hours post infection (186, 187). There is no clear evidence that TNF is actually secreted during viral infection *in vivo*; however, stimulation of macrophages with IFN-γ is sufficient to induce membrane TNF-expression to levels that induce the cytolysis of virus-infected cells, and *in vivo*, it may be the cell-associated form of TNF that is the biologically relevant mediator of the antiviral properties of TNF (127, 188). Another cytokine, IFN-γ, also has direct antiviral activity and enhances the antiviral properties of TNF (179, 189). Ramsay *et al* (189) have proposed that *in vivo*, CD8$^+$ T cells act primarily to focus TNF and IFN-γ at sites of virus replication which then act to clear viral infection. When euthymic mice, sublethally irradiated, were infected with recombinant vaccinia viruses expressing either IFN-γ or TNF, the potent antiviral activity of the cytokines led to rapid virus clearance, strongly supporting a direct antiviral role for IFN-γ and TNF *in vivo* (186, 190).. Ramsey *et al* further postulate that the activity of these factors, perhaps in synergy, not only controls virus replication, but also induces an antiviral state in uninfected neighboring cells.

The TNFs have also been associated with various disease pathologies. In addition to cachexia and endotoxemia/toxic shock which have already been described, TNF has been implicated in the development of insulin resistance, the mediation of allograft rejection and graft versus host disease, the triggering of adult respiratory distress syndrome, the development of reperfusion injury, joint destruction, and the pathogenesis of diseases of the central nervous system including multiple schlerosis, meningitis, brain tumors and cerebral edema (176, 191-193). Moreover, soluble TNFRs, TNFR immunoadhesions, or antibodies against TNF have been shown to protect against toxic shock/endotoxemia and arrest the development of collagen arthritis in mice (194-199). Soluble versions of the extracellular ligand binding regions of the TNFRs naturally occur within body fluids, and are thought to regulate the activities of TNF as TNF antagonists, as TNF carriers, or alternatively a TNF/sTNFR complex might increase the half-life of TNF (164, 194, 200, 201).

TNFRs and TNF Signal Transduction Cascades

Many of the biological responses to TNF and LTα are mediated through ligand oligomerization of either the type I, p55/p60 TNFR or the type II, p75/p80 TNFR (66, 156, 164, 173). Both the mouse and human type I and type II TNFRs have been cloned (64, 66, 156, 164, 173, 202-204), and both receptors bind TNF and LTα with high affinity dissociation constants ranging from 50 pM to 1 nM (167, 205-207). TNFRs are present on virtually all cell types with the exception of eythrocytes, with a constitutive level of expression of the type I, p55 TNFR (approximately 1000 copies/cell), and an inducible level of type II, p75 TNFR expression (206-210). Although the mouse p55 TNFR and the human p55 and p75 TNFRs recognize both mouse and human TNF, the mouse p75 TNFR shows strong species specificty for mouse TNF (211). Finally, the crystal structure of the extracellular domain of the human p55 TNFR with LTα has been solved (212). The extracellular region forms an elongated end-to-end structure of four similarly folding domains defined by the CRDs, and contact sites with LTα are mainly confined to the second CRD, and to a lesser extent the first and third CRDs. The trimeric ligand binds multiple receptor monomers, which leads to receptor aggregation or clustering.

A number of strategies have been used to determine which TNFR activates and controls the various signaling and biological responses to TNF, including triggering receptors with receptor-specific antibodies, inhibiting TNF responses with receptor antagonist antibodies, using human TNF to effect responses mediated by the mouse p55 TNFR, creating TNF mutants with exclusive specificity for either receptor, expressing the TNFRs in TNFR-deficient cell lines, and studying the effects of receptor knock-out in transgenic mice. The p55 TNFR mediates many of the biological responses associated with TNF: NF-κB activation, upregulation of HLA, ICAM, VCAM, and CD44, IL-6 production, neutrophil respiratory burst, growth inhibition and cytotoxicity, induction of manganous superoxide dismutase which protects against cytolysis, prostaglandin E_2 synthesis, upregulation of EGF receptor mRNA, fibroblast proliferation, and leukocyte adhesion to the endothelium (206, 208, 210, 213-221). The p75 TNFR appears to mediate thymocyte and cytotoxic T lymphocyte (CTL) proliferation and in certain cell lines may activate NF-κB (206, 213, 222). Some researchers report that the p75 TNFR enhances p55 TNFR cytolysis and potentiates neutrophil and endothelial cell activation mediated by the p55 TNFR (223-225). A passive model of p75 TNFR action has been proposed in which the p75 TNFR increases the local concentration of TNF at the cell surface through rapid ligand association and dissociation which in turn regulates the rate of TNF association with the p55 TNFR, a mechanism referred to as ligand passing (226). In the past, there have been conflicting reports whether the p75 TNFR alone is capable of inducing cytolysis (211, 227-229). The evidence is mounting, however, that support a model of direct p75 TNFR-mediated signaling, including NF-κB activation and apoptosis of mature T cells (222, 230). Moreover, although the p55 TNFR itself can induce cytolysis, both receptors may be required for apoptotic death in some cells; in other cells, the two receptors may mediate apoptosis through different signaling pathways (229, 231, 232). Finally, the membrane-bound form of TNF appears to be the principal activating ligand for the p75 TNFR, and activation of p75 TNFR by membrane bound TNF leads to different responses than with soluble TNF, such as resistance to cytolysis (233).

The characterization of events immediately following receptor activation were hampered by the fact that the cytoplasmic domains of the TNFRs possess no apparent catalytic activity and share no homology with other receptors whose signaling pathways have been characterized, and downstream signaling events were described before the identification of factors associating with the TNFRs. Three main TNF/LTα signal transduction pathways have been described (158, 165, 167, 205, 209, 234, 235), and an overview of TNF signaling is shown in Figure I-3. TNF activates PLA$_2$ resulting in the production of arachidonic acid, 5-HPETE, leukotrienes and prostaglandins, which account, in part, for the pro-inflammatory properties of TNF (236, 237). Moreover, PLA$_2$ activation appears to be important in TNF-induced cytolysis, and the arachidonic acid metabolite 5-HPETE mediates effects on *c-fos* gene expression (237-240). The second major pathway involves the activation of a phosphatidylcholine-specific phospholipase C, with the production of the second messenger diacylglycerol, an activator of protein kinase C (241). Protein kinase C in turn phosphorylates a number of proteins eventually leading to transcriptional activation (165). Finally, a third pathway involves the activation of a neutral sphingomyelinase which hydolyzes sphingomyelin generating the second messenger ceramide, leading to the phosphorylation of a number of downstream effectors such as ceramide-activated protein kinase, PKC-ζ, c-raf-1 kinase, MAP kinase, and IκB, and culminating in transcriptional activation (234, 242-248). Moreover, an endosomal acidic sphingomyelinase has been implicated in the activation of NF-κB (249). TNF also induces the activation of JNK protein kinases and the AP-1 transcription factor, and the JNK protein kinases have been implicated in the induction of apoptosis (250-252). The different TNF signaling pathways may overlap; for example, MAP kinase induction might lead to PLA_2 activation (234). Moreover, recently it has been suggested that NF-κB activation and ceramide production might represent different arms of the signaling response, with ceramide production being a consequence of the activation of ICE-like proteinases in an apoptotic signal and not an upstream effector of NF-κB activation in the signaling cascade (253).

TNFR-associated proteins were first described for the p75 TNFR, and named TRAF1 (TNFR-associated factor) and TRAF2 (254). The proteins share homology in a region within their C-termini, designated the TRAF domain, which is involved in oligomerization and receptor binding (255). TRAF2 contains N-terminal zinc finger and RING finger motifs, and both TRAF1 and TRAF2 can form homodimers and heterodimers. TRAF2 associates directly with the cytoplasmic domain of the p75 TNFR, and TRAF1 interacts indirectly with the receptor by forming a heterodimer with TRAF2. A CD40 binding protein, TRAF3/CRAF1, also possesses a TRAF domain (256). TRAF2, a common mediater of p75 TNFR, CD30 and CD40 signaling, is expressed in a broad variety of tissues, whereas TRAF1 is limited to spleen and thymus. Over-expression of TRAF2, but not TRAF1 or TRAF3, leads to NF-κB activation (257, 258). Two additional proteins, c-IAP1 and c-IAP2, are recruited to the p75 TNFR via interaction with a TRAF2-TRAF1 heterocomplex (126). c-IAP1 and c-IAP2 are cellular homologs of the baculovirus inhibitor of apoptosis protein (IAP), and both cellular and viral IAPs contain N-terminal BIR (baculovirus IAP repeat) motifs and a C-terminal RING finger. The c-IAPs appear to associate with TRAF2-TRAF1 complex via their BIR motifs.

The Epstein-Barr virus LMP-1 protein binds to cellular proteins, named EB16 and LAP1 (259). EB16 is identical to TRAF1 and LAP1 is the same as TRAF3. Association of LAP1 and EB16 with the Epstein Barr virus transforming protein, LMP1, causes all three to localize to the plasma membrane, which may be important in transformation. Moreover, TRAF3 also associates with the p75 TNFR, CD40 and the LTβ receptor.

Mutant TNFRs with truncations in the C-terminal cytoplasmic domains showed that discrete regions within the p55 TNFR generate distinct signals and identified a unique 80 amino acid cytoplasmic motif, termed the death domain, important in signaling cell death, mediating antiviral effects and activating an acidic sphingomyelinase (246, 249, 260). A similar motif required for apoptosis, is present in the cytoplasmic domain of Fas (261). Both p55 TNFR and Fas self-associate via their death domains and, using yeast two hybrid selection, a TNFR-associated protein, TRADD, was identified which possesses a death domain and which specifically interacts with the p55 TNFR via the death domains (262, 263). Over-expression of TRADD induces two major TNF responses, apoptosis and NF-κB activation, but the poxviral inhibitor of ICE, crmA, inhibits only apoptosis and not NK-κB activation induced by TRADD overexpression. Moreover, TRADD directly interacts with TRAF2 to activate NF-κB, and interacts with FADD, a protein identified because of its association with Fas, to enhance its association with the p55 TNFR leading to the induction of apoptosis (258). A serine kinase, RIP, identified by yeast two hybrid cloning associates with Fas and the type I TNFR; overexpression of RIP leads to cell death (264). Two-hybrid selection has identified two other proteins which associate with the type I TNFR: TRAP-1 that shows homology to hsp90, and a second protein which binds upstream of the TNFR death domain, although the significance of these proteins for TNF signaling has yet to be established (265, 266). Moreover, ligand induced serine/threonine and serine kinase activities have been identified that associate with the p55 TNFR, although it awaits to be determined whether TRADD or another TNFR associated protein is a substrate for either of these activities (267, 268).

Lessons from Knockout Mice

Both p55 TNFR- and p75 TNFR-knockout mice have been constructed, and considering the pleiotropy of TNF action, the p55 TNFR-null and p75 TNFR-null mice have surprisingly normal phenotypes, including thymocyte and lymphocyte development (269-271). The p55 TNFR-null mice, however, are more resistant to endotoxic shock, succumb to *Listeria monocytogenes* infection, and do not develop germinal centers within peripheral lymphoid organs, whereas the p75 TNFR-null mice exhibit a dramatic decrease in tissue necrosis following subcutaneous injection with TNF, in agreement with the p75 TNFR promoting inflammation (269-272). In sharp constrast with the receptor story, LTα-null mice exhibit a complete absence of detectable lymph nodes or Peyer's patches although thymic development appears normal, suggesting an essential role for LTα in the development of peripheral lymphoid organs, independent of p55 or p75 TNFR interaction (273). Furthermore, LTα knockout mice are also deficient in germinal center formation (274).

LTβ and the LTβ Receptor

In addition to the soluble LTα which activates the p55 and p75 TNFRs, there is also a membrane associated form of LTα which is anchored to the cell surface via its association with LTβ, a TNF family member that is expressed in lymphoid and hematopoietic tissue (275-277). The predominant form of the LT membrane complex is $LT\alpha_1\beta_2$ heterotrimer, with $LT\alpha_2\beta_1$ a minor species, and the LTα/LTβ complex recognizes the LT-β receptor, expressed in liver, lung, kidney, heart, spleen and lymph node tissue (278, 279). Because of the presence of lymph nodes in the TNFR knockout mice and the dramatic phenotype observed with the LTα knockout mouse, the LTβ receptor has been implicated in mediating the development of peripheral lymph nodes, although the possibility remains that another receptor for the LT complex exists (158, 173).

Fas and Fas Ligand

In 1989, two research groups independently reported the isolation of monoclonal antibodies capable of killing cells upon binding to the cell surface antigens, and the subsequent cloning of the surface antigens recognized by these antibodies, designated Fas and APO-1, revealed they were the same molecule (280-283). Although it initially had been thought that Fas might be a TNF receptor, cloning revealed instead that Fas was a novel member of the TNFR superfamily which mediates apoptosis, is involved in the alternative, perforin-granzyme independent pathway of CTL cytotoxicity, and downregulates immune responses (284, 285). Fas is expressed on activated T cells, B cells, monocytes and neutrophils (156, 285). The Fas ligand (FasL), a member of the TNF superfamily, has been cloned, and is expressed on activated splenocytes and thymocytes, consistent with its involvement in T cell-mediated cytotoxicity (286). Moreover, like TNF, soluble FasL expressed from COS cells is a trimer (287). The spontaneous mouse mutations, *lpr* and *gld* are loss of function mutations of Fas and FasL respectively, and the phenotype of mice bearing these mutations is a generalized lymphoproliferative disease with lymphoadenopathy and splenomegaly, and the production of large quantities of IgG and IgM antibodies and autoantibodies, suggesting that the Fas and FasL system plays a role in normal lymphocyte death (285). Expression of FasL in non-lymphoid cells such as fibroblasts is sufficient to make such cells cytotoxic (285, 286). Fas mediated cytolysis is independent of extracellular calcium, primarily involves cytoplasmic rather than nuclear events, and does not require protein synthesis (284, 285). Moreover, ceramide may be a second messenger of Fas signaling (285). Fas-mediated cell death is inhibited in part by overexpression of Bcl-2 and completely inhibited by expression of cowpox crmA or coexpression of Bcl-2 and its binding protein BAG-1 (93, 288-290).

The C-terminal cytoplasmic region of Fas contains the death domain motif also present in the p55 TNFR, and Fas interacts with two other death domain containing proteins, FADD/MORT-1 and RIP (264, 291, 292). Overexpression of FADD induces apoptosis which is inhibitable by crmA. Moreover, FADD can associate with the p55 TNFR via association with TRADD, although the p55 TNFR/TRADD/FADD complex appears to be unstable (258). The N-terminal region of RIP shares homology with protein kinases including $p56^{lck}$, while the C-terminal region contains a death domain, and transient expression of RIP causes cells to undergo apoptosis (264). Weak association of RIP with the p55 TNFR has been observed (264). It awaits to be determined what the exact functions of FADD and RIP are in Fas signaling.

Although Fas and p55 TNFR have similar death domains, there are differences between Fas- and TNF-mediated cell lysis: Fas-induced cell death is quicker than that induced by the p55 TNFR and is not blocked by manganese superoxide dismutase, metallothionen, plasminogen activator type 2, A20 or mitochondrial inhibitors (293-296). Moreover Fas does not induce the activation of NF-κB (294).

The antiviral effects of Fas have not been well characterized. Influenza virus induces the expression of the Fas gene in infected cells, which, in turn, may have an antiviral effect on influenza infection (297), but clearance of infection with lymphocytic choriomeningitis virus, vesicular stomatitis virus and Semliki forest virus in mice is independent of Fas (298). However, anti-Fas antibodies, but not antibodies against other members of the TNFR superfamily, induced apoptosis of T lymphocytes from HIV-infected individuals (299). Moreover, poxviruses may use several strategies to evade a Fas antiviral response. The cowpox crmA protein, described previously, inhibits Fas-mediated apoptosis (93), and infection of B and T lymphoblast cell lines with vaccinia virus strain WR (*crmA* homolog$^+$) but not strain Copenhagen (*crmA* homolog$^-$) rendered the cells resistant to Fas-mediated apoptosis (300).

CD40 and CD40 Ligand

CD40 is a glycoprotein expressed on B cells, thymic epithelium, monocytes, dendridic cells and at low levels on some T cells, whereas the ligand for CD40 (CD40L) is expressed on activated T cells (156, 301). In B cells, CD40 appears to regulate proliferation and apoptosis, immunoglobulin class-switching, the generation of memory B-cells, cytokine gene expression and cell adhesion (156, 301). In T-cells, CD40L stimulates proliferation and cytokine secretion and induces expression of several cell-surface activation antigens, and in monocytes, CD40L stimulates TNF, IL-6 and IL-8 production. TRAF2 appears to be a common mediator of both p75 TNFR and CD40 signaling, and a truncated TRAF lacking a RING finger domain was a dominant negative inhibitor of CD40 activation of NF-κB (257). TRAF3/CRAF1/CAP-1 is a protein shown to associate with CD40 via its C-TRAF domain (302, 303). Similar to TRAF2, TRAF3 also contains RING finger and zinc finger motifs near its N-terminus. The Epstein-Barr virus LAP1, which is homologous to TRAF2, coimmunoprecipitates with CD40 possibly by association with TRAF3 (259).

Patients with mutations or deletions in CD40 L exhibit X-linked hyper-IgM syndrome and show increased sensitivity to infection (301). Moreover, CD40 L has been shown to have potent antiviral activity. Recombinant vaccinia viruses expressing CD40 L were dramatically attentuated even in mice lacking T or B cells (304). Moreover, when L929 cells were pretreated with membrane preparations containing CD40L and then infected with HSV-1, the resulting virus yields were reduced comparable to that observed when the cells were pretreated with mouse TNF (304).

Other Receptors and Ligands within the TNFR and TNF Superfamilies

CD30 is a 120 kDa surface antigen widely used as a clinical marker for Hodgkin's lymphoma, although it is also expressed on transformed B- and T- cells, and its expression can be induced on untransformed T cells (156). CD30 ligand is expressed on activated T cells and macrophages, and enhances the proliferation of CD3-activated T cells but induces cell death in a CD30$^+$ lymphoma cell line (305).

CD27, a disulphide-linked homodimer of 55 kDa subunits, is expressed on the majority of medullary thymocytes and peripheral blood T cells, and on a subpopulation of

B and NK cells, although activation of T cells leads to the upregulation of *CD27* gene expression and the secretion of a 32 kDa soluble form of the receptor (156). Helper T cells often express CD27, whereas memory T cells lack CD27. The ligand for CD27 is expressed by activated T cells, Epstein-Barr virus transformed B cells and pre-B lines, and CD27 ligand acts to induce the proliferation of activated T cells and enhances the generation of cytolytic T cells (306).

4-1BB is a 35 kDa receptor expressed on activated T cells, and its ligand is also expressed on activated T-cells (307). 4-1BB ligand stimulates the proliferation of activated T cells, but may also trigger cell death in some T cell clones.

OX40 and OX40 ligand are 50 kDa and 34 kDa proteins expressed on activated $CD4^+$ T cells which act to costimulate T cell proliferation and induce IL-4 secretion (308). Moreover, OX40 ligand is identical to gp34 whose expression on HTLV-1-infected human leukemic T cells is regulated by the *tax* gene.

Finally, TRAIL is a newly identified TNF family member whose receptor has not been determined and it is expressed in a wide variety of tissues including spleen, thymus, prostrate, ovary, small intestine, colon, peripheral blood lymphocytes, placenta, lung, kidney and pancreas (159). Both secreted and surface expressed TRAIL induce apoptosis in a wide variety of cells.

THESIS OBJECTIVES

The purpose of this work was to elucidate the mechanisms in which M-T2 protein contributes to myxoma virus virulence, to characterize biochemical properties of M-T2, and to determine whether TNF is a biologically relevant target for M-T2 action. Preliminary work involved characterizing the species specificity of rabbit cell lines to the cytolytic activities of TNF and investigating the species specificity of M-T2 inhibition of TNF. To test whether TNF has antiviral effects on poxviruses *in vitro*, the ability of TNF to inhibit myxoma virus and vaccinia virus replication in tissue culture was examined. Various biochemical properties of M-T2 were studied, including its monomeric/dimeric nature, native size, and glycosylation. The kinetics of *M-T2* transcription, protein expression and secretion were examined in detail. To show that rabbit TNF is a relevant target for M-T2 protein, dissociation constants for the M-T2/TNF interaction were determined. Moreover, the molar amounts of both monomeric and dimeric M-T2 required to inhibit the cytolytic activites of TNF were calculated and compared with the molar amounts of soluble TNFRs required to inhibit TNF. Mutant M-T2 proteins containing C-terminal truncations or deletions of the cysteine rich domains sharing homology with cellular TNFRs were constructed in vaccinia virus vectors, and were tested for their ability to co-immunoprecipitate and inhibit rabbit TNF. During the course of these investigations, it was discovered that M-T2 inhibits apoptosis in myxoma infected CD4$^+$ T cells (RL-5 cells). Therefore, recombinant myxoma viruses were constructed expressing the M-T2 C-terminal truncations instead of full length M-T2 protein, and the ability of these viruses to replicate in RL-5 cells and to inhibit the apoptosis of myxoma-infected RL-5 cells was measured.

Figure I-1. Bam HI Map of the Myxoma Genome

The Bam HI map of the myxoma genome is shown, with vertical lines indicating Bam HI restriction enzyme sites, and arrows designating the locations of the virulence factors M-T2, M-T5, M-T7, Serp-1, MGF and M11L, and the length of the terminal inverted repeats (TIR).

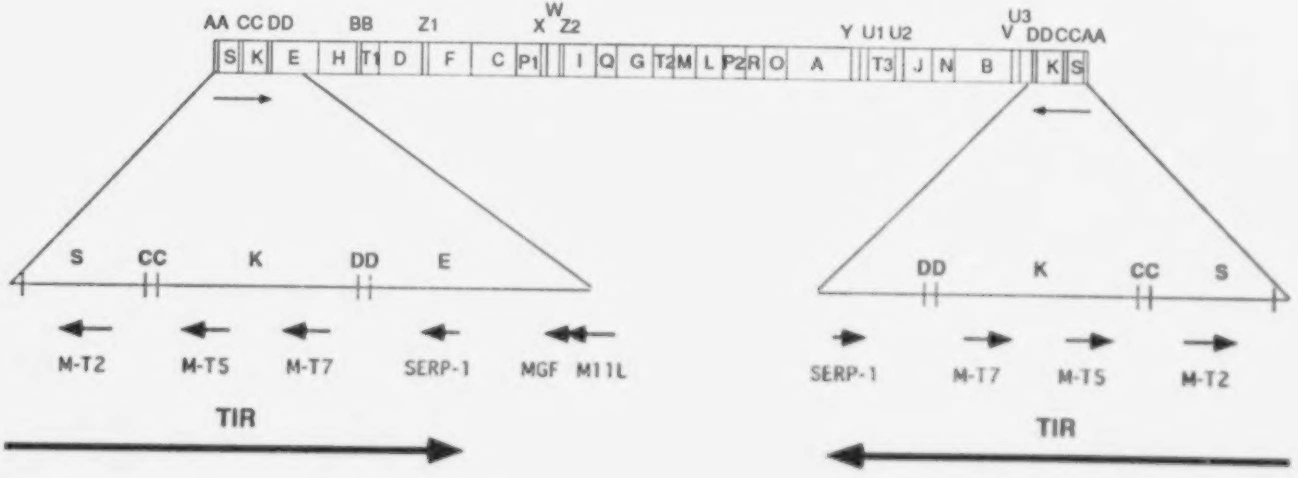
AA CC DD
BB
Z1
X W Z2
Y U1 U2
U3 V DD CC AA
S K E H T1 D F C P1 I Q G T2 M L P2 R O A T3 J N B K S
S
CC
K
DD
E
M-T2
M-T5
M-T7
SERP-1
MGF
M11L
TIR
DD
K
CC
S
SERP-1
M-T7
M-T5
M-T2
TIR

Figure I-2. Cellular and Poxviral Members of the TNF Receptor Superfamily

Characteristic features of the poxvirus TNF receptor homologues including N-terminal leader sequences, four cysteine rich repeats,and C-terminal domains are compared with each other, the two human TNF receptors and other members of the TNFR superfamily.. Conserved cysteines are outlined (|), whereas (⊤) designate frameshifts and (⫯) designates a stop codon in the Vaccinia A53R and C22L sequences.

HOMOLOGIES WITHIN THE TNF RECEPTOR SUPERFAMILY

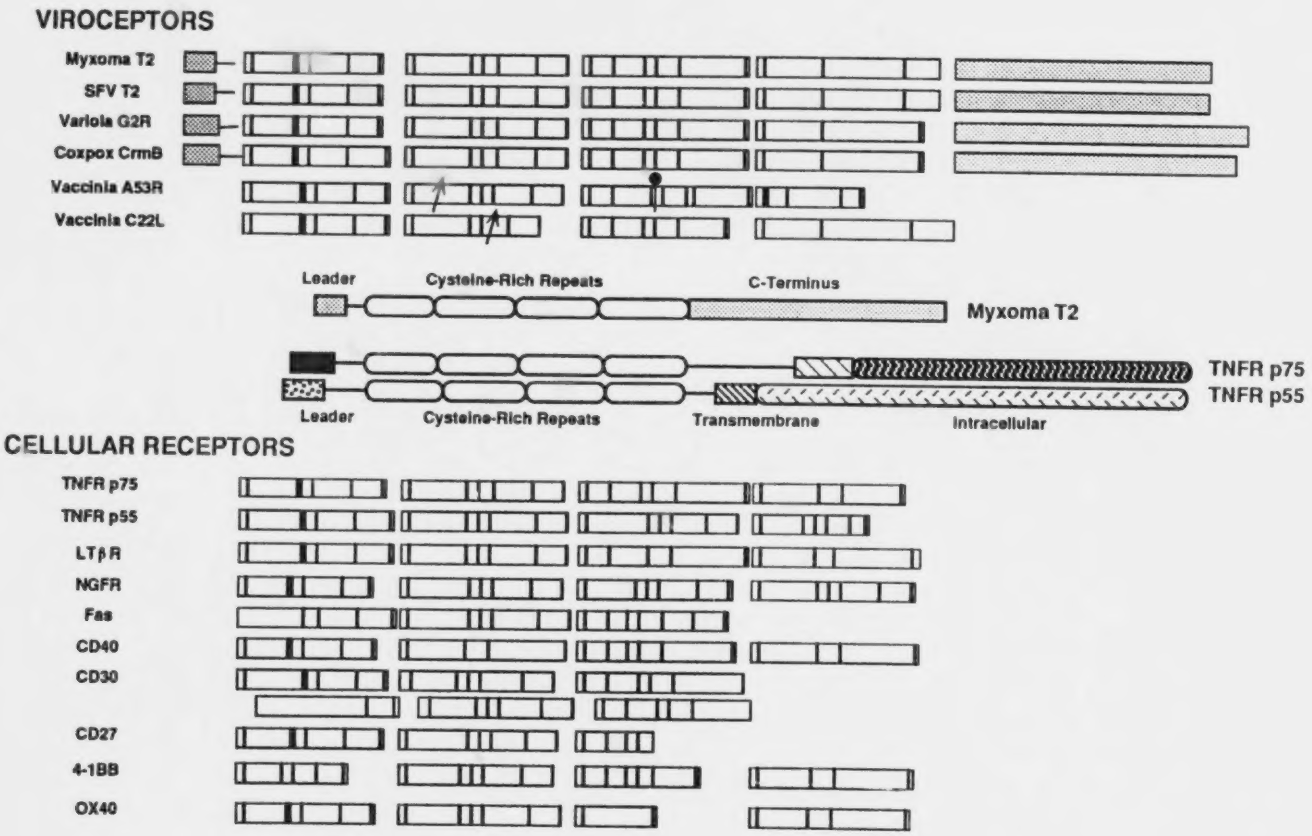
VIROCEPTORS
Myxoma T2
SFV T2
Variola G2R
Coxpox CrmB
Vaccinia A53R
Vaccinia C22L
Leader
Cysteine-Rich Repeats
C-Terminus
Myxoma T2
TNFR p75
TNFR p55
Leader
Cysteine-Rich Repeats
Transmembrane
Intracellular
CELLULAR RECEPTORS
TNFR p75
TNFR p55
LTβR
NGFR
Fas
CD40
CD30
CD27
4-1BB
OX40

Figure I-3. Overview of TNF Signaling

Ligand-induced p55 and p75 TNF receptor clustering leads to the association of various factors with the receptor, and the subsequent activation of various signal transduction pathways, including the activation of PLA_2 (phospholipase A_2) which leads to the production of arachidonic acid, phospholipase C (PLC) which hydrolyzes phosphatidyl choline to the second messenger diacyl glycerol and phosphoryl choline, and a neutral sphingomyelinase which generates the second messenger ceramide.

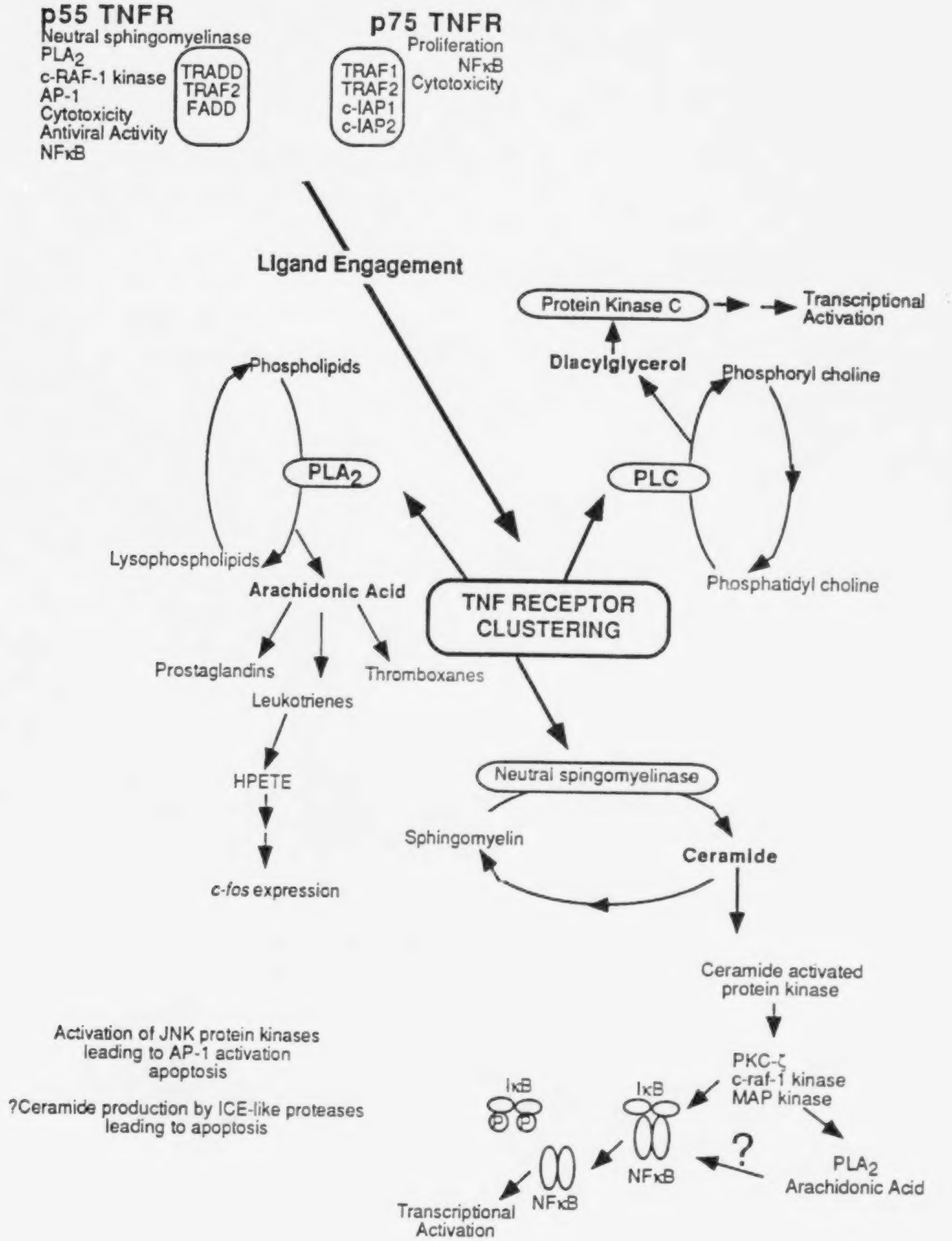
p55 TNFR
Neutral sphingomyelinase
PLA$_2$
c-RAF-1 kinase
AP-1
Cytotoxicity
Antiviral Activity
NFκB
TRADD
TRAF2
FADD
p75 TNFR
Proliferation
NFκB
Cytotoxicity
TRAF1
TRAF2
c-IAP1
c-IAP2
Ligand Engagement
Protein Kinase C
Transcriptional Activation
Diacylglycerol
Phosphoryl choline
Phospholipids
PLA$_2$
PLC
Lysophospholipids
Arachidonic Acid
Phosphatidyl choline
TNF RECEPTOR CLUSTERING
Prostaglandins
Leukotrienes
Thromboxanes
HPETE
c-fos expression
Neutral spingomyelinase
Sphingomyelin
Ceramide
Ceramide activated protein kinase
Activation of JNK protein kinases leading to AP-1 activation apoptosis
?Ceramide production by ICE-like proteases leading to apoptosis
PKC-ζ
c-raf-1 kinase
MAP kinase
IκB
IκB
NFκB
?
PLA$_2$
Arachidonic Acid
NFκB
Transcriptional Activation

Table I-1. Poxviruses of Vertebrates

Family: Poxviridae; Subfamily Chordopoxviridae

Genus	Species	
Orthopoxvirus		Natural host
	Variola	*Homo sapiens*
	Cowpox	? wild rodents
	Ectromelia	? voles, laboratory mouse
	Vaccinia*	?
	Buffalopox, camelpox, monkeypox, rabbitpox, raccoon pox, tatera pox	
Suipoxvirus	Swinepox	
Leporipoxvirus		Natural host
	Shope fibroma	*Sylvilagus floridanus*
	Myxoma:	
	(California)	*Sylvilagus bachmani*
	(South America*))	*Sylvilagus brasiliensis*
	Squirrel fibroma	*Sciurus carolinensis*
	Western squirrel fibroma	*Sciurus griseus*
	Hare fibroma	*Lepus capensis*
Avipoxvirus	Canary pox, fowlpox*, junco pox, pigeon pox, quail pox, sparrow pox, starling pox, turkey pox	
Capripoxvirus	Goatpox, sheeppox*, lumpy skin disease	
Parapoxvirus	Chamois contagious ecthyma, Orf*, pseudocowpox, stomatitis papulosa	
Molluscipoxvirus	Molluscum contagiosum	
Yantapoxvirus	Tanapox*, Yaba	

* Protypical member

Table I-2. Poxviral Gene Products that Counteract Host Defenses

NAME	VIRUS	FUNCTION	HOST HOMOLOG	"LIGAND"/ SUBSTRATE
VIROKINES:				
Inhibition of complement				
C21L	Vaccinia	Blocks complement activation	Complement binding protein	C3b and C4b
B5R	Vaccinia	Required for extracellular virus formation	Complement Control Proteins	
B6R	Variola			
ps/hr	Rabbitpox			
Growth factor homologs				
VGF (C11R)	Vaccinia/	Stimulates cell growth of quiescent cells	EGF/TGFα	Growth factor receptors
D4R	Variola			
MGF	Myxoma			
SFGF	SFV			
A2R	orf virus		VEGF	
VIROCEPTORS:				
TNFR homologs				
S-T2	SFV	Binds and sequesters host TNF	Type I and II TNFRs	TNF
M-T2	Myxoma			
Crm B	Cowpox			
G2R/G4R	Variola			
IFN-γ receptor homologs				
M-T7	Myxoma	Binds and sequesters host IFN-γ	IFN-γ R	IFN-γ
S-T7	SFV			
T7 homolog	Ectromelia			
B8R	Vaccinia			
B8R/B9R	Variola			
C6L	Swinepox			
IFN-γ binding proteins present in rabbitpox, cowpox, camelpox				
IFN-α/β receptor homologs				
B18R	Vaccinia	Binds type I IFNs	Type I IFN R	IFN α/β
B17R/B20R	Variola			
Type I IFN binding proteins present in cowpox, elephantpox and camelpox				
IL-1β receptor homologs				
B15R	Vaccinia Cowpox	Sequesters IL-1β and blocks its functions	IL-1β R	IL-1β
IL-8 receptor homologs				
C3L	Swinepox	Displays homology with the low affinity IL-8 receptor	IL-8 R, low affinity	
K2R	Swinepox	Displays homology with the low affinity IL-8 receptor ?Chemokine binding	IL-8 R, low affinity	? Chemokines

TABLE I-2 (cont'd)

NAME	VIRUS	FUNCTION	HOST HOMOLOG	"LIGAND"/ SUBSTRATE
INHIBITORS OF CYTOKINE ACTIVATION:				
SPI-2/crmA				
SPI-2/crmA B13R	Cowpox Vaccinia-WR Variola Rabbitpox	Blocks inflammation in the chicken chorioallantoic membrane Inhibitor of IL-1β converting enzyme (ICE) Inhibits Fas- and TNF-induced apoptosis	Serpins	ICE or an ICE-like proteinase
SPI-1	Homologs in cowpox, vaccinia, rabbitpox, variola	? Inhibits apoptosis	Serpins	Unidentified serine proteinase
SPI-3	Homologs in vaccinia, cowpox, rabbitpox, variola	Prevents cell-cell fusion	Serpins	?Unidentified serine proteinase
SPI-4 Serp-1	Myxoma	Inhibits the inflammatory response	Serpins	?Extracellular serine proteinase
OTHER VIRULENCE FACTORS:				
Inhibitors of IFN				
E3L	Vaccinia	Inhibits IFN-induced PKR IFN resistance Prevents apoptosis of fibroblasts	PKR homolog	Binds dsRNA
K3L C3L	Vaccinia Variola	Prevents the phosphorylation of eukaryotic initiation factor 2α IFN resistance	eIF-2α homolog	Substrate for PKR
Inhibitors of apoptosis:				
M-T5	Myxoma	Prevents shutoff of protein synthesis and apoptosis in lymphocytes		
CHOhr	Cowpox	Allows for productive vaccinia virus infection of CHO cells Prevents shutoff of protein synthesis and delays apoptosis		
M-T2	Myxoma	Inhibits apoptosis and allows for productive infection in a rabbit lymphocyte line	TNFR superfamily	?Fas, TNF/LTα, CD30, TRAIL

TABLE I-2 (cont'd)

NAME	VIRUS	FUNCTION	HOST HOMOLOG	"LIGAND"/ SUBSTRATE
M-11L	Myxoma	Virulence factor that prevents infiltration of inflammatory cells Inhibits apoptosis and allows for productive infection in a rabbit lymphocytes and monocytes	?	?
Late gene product(s)	Myxoma	Loss of MHC class I antigens	?	?
?	Myxoma	CD4 downregulation and $p56^{lck}$ dissociation in $CD4^+$ T cells	?	?
Homologs of host metabolic enzymes				
SalF7L	Vaccinia	Synthesis of steroid hormones Virulence factor	3β-hydroxysteroid dehydrogenase	

Table I-3. Viral Proteins which Inhibit Apoptosis

VIRUS	PROTEIN	MECHANISM
Adenovirus	E1B 19K	Blocks p53
	E1B 55K	Sequesters p53
	E3-14.7K	Prevents TNF cytolysis Blocks activation of $cPLA_2$
	E3-10.4K/14.5K	Prevents TNF cytolysis and activation of $cPLA_2$
Simian virus 40	T antigen	Inactivates p53
Human papilloma virus	E6	Inactivates p53
Epstein-Barr virus	LMP1	Upregulates Bcl-2 expression
	BHRF1	Bcl-2 homolog
Baculovirus	p35	?
	iap	? Cellular homologs cIAP1 and 2 associate with Type II TNFRs
Hepatitis B virus	HB_x	Forms complexes with p53
Herpes simplex virus	$\gamma_1 34.5$	Prevents shutoff of protein synthesis
Cowpox virus	crmA	Inhibits ICE or an ICE-like proteinase
	CHOhr	?
Vaccinia virus	E3L	Inhibits PKR
African swine fever virus	LMW5-HL	Bcl-2 homolog
Myxoma virus	M-T5	Prevents shutoff of protein synthesis
	M-T2	?
	M11L	?

Table I-4. The TNF Ligand and Receptor Superfamilies

RECEPTOR Name	 Cellular expression	 Associated proteins	LIGAND Name	 Source	FUNCTION
Type I/p55 TNFR	ubiquitous	TRADD TRAF2 FADD	TNF	Macrophages, T cells, many cells	Immune responses Inflammation Apoptosis
			LTα	T and B cells	
Type II/p75 TNFR	ubiquitous	TRAF1 TRAF2 c-IAP1 c-IAP2	TNF	Macrophages, T cells, many cells	Immune responses Inflammation Cell survival and proliferation Apoptosis
			LTα	T and B cells	
LTβR	Viscera, spleen and lymph node tissue	TRAF3	LTα/β complex	lymphoid and hematopoietic cells	Formation of peripheral lymphoid tissue ?Apoptosis
Fas/APO-1	T cells Reproductive tissue	FADD/ MORT-1 RIP	Fas ligand	activated splenocytes and thymocytes	Lymphoproliferation T-cell mediated cytotoxicity Apoptosis
CD40	T cells Mast cells	TRAF3/CRAF TRAF2	CD40 ligand	activated T cells	Ig class switching Cell survival Antiviral responses
CD30	T cells Monocytes	TRAF1 TRAF2 TRAF3	CD30 ligand	activated T cells and macrophages	T cell proliferation and B cell maturation Apoptosis
CD27	B cells, thymic stroma, T cells		CD27 ligand	activated T cells	antigen-driven T-cell activation and proliferation
4-1BB	Lymphoid cells and stromal lines		4-1BB ligand	activated T cells	Antigen-presenting cell-driven T cell activation and proliferation
OX-40	T cells		OX-40 ligand	activated T cells	T cell activation
?			TRAIL	Viscera, spleen and lymphocytes	Apoptosis

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CHAPTER II - MATERIALS AND METHODS

REAGENTS

Viruses and Cells

Vaccinia virus (strain WR) and myxoma (strain Lausanne) were obtained from the ATCC. VV-601 containing the *Escherichia coli* lac Z gene inserted into the vaccinia thymidine kinase (*tk*) locus has been described previously (1). The myxoma virus (strain Lausanne) derivative, vMyxlac, which contains the *E. coli* lacZ gene inserted between the myxoma growth factor and M9 genes (2), vMyxt2g in which both copies of T2 are disrupted (3); vMyxt2r, in which both copies of T2 disrupted in vMyxt2g have been reconstituted (4); vMyxSERP$^-$, in which both copies of the *SERP-1* gene have been inactivated (1); and vMyxM11L$^-$, in which the *M11L* gene has been inactivated, have all been described elsewhere (1, 2).

Viruses were propagated in monkey BGMK cells (obtained from S. Dales) grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS; Gibco). Rabbit RK-13 cells and SIRC cells (obtained from the ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco). Mouse L929-8 cells (provided by L. Guilbert) were described previously (5), and were grown in Iscove's modified Eagle's medium(IMDM) supplemented with 10% FBS. U937 cells (obtained from L. Guilbert), a hunan monocyte line, were grown in RPMI media supplemented with 10% FBS. Rabbit RL-5 cells (CD4$^+$-T cell lymphoma cells obtained from the NIH AIDS Research and Refrence Reagent Program) were cultured in RPMI media supplemented with 10% FBS.

CONSTRUCTION OF RECOMBINANT VIRUSES

The various recombinant viruses geneated for this thesis are diagrammed in Figure II-1. All recombinant techniques followd standard procedures outlined in (6).

Expression of Myxoma T2 and TNFs from Recombinant Vaccinia Viruses

The myxoma T2 gene sequence contains an Nla III site (CATG↓) at the translation start site but not in the coding sequences (3). A 1060 bp Nla III fragment containing the complete T2 open reading frame was subcloned into the iso-accepting Sph I site of pMTL21, generating pMTN-6. A 1117 bp Hind III/Bam HI fragment was then subcloned into the HindIII /Bam HI sites of the vaccinia expression plasmid pMJ602 (7), to create pMJ602MT2, allowing T2 to be inserted into the thymidine kinase *(tk)* gene of vaccinia virus under the control of a strong, synthetic late promoter.

Rabbit TNF was cloned by the amplification of a cDNA copy generated by reverse transcription of rabbit monocyte mRNA. Peripheral blood lymphocytes were isolated by layering rabbit blood over an equivalent volume of Ficoll:Paque and centrifuging at 400 x g for 40 minutes. The lymphocyte fraction was collected, washed twice with phosphate buffered saline (PBS), resuspended in RPMI supplemented with 10% FBS, and incubated 4 hours at 37°C. The adherent cells, enriched for monocytes, were incubated a further 2 hours in the presence of 1 μg/ml lipopolysaccharide (LPS, Sigma). RNA was prepared by the guanidinium/cesium chloride method (6) except the cesium chloride pelleted RNA was dissolved in 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), extracted with phenol:chloroform (1:1) and ethanol precipitated. The first strand of cDNA was synthesized by incubating 1 μg RNA with 50 pmoles of primer (5' TGACTGTGCAGGCCTACACCACC) hybridizing to rabbit TNF 3' untranslated sequences (8), 1mM dXTPs, and 200 units Superscript reverse transcriptase (GibcoBRL) in 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 4 mM $MgCl_2$, 0.01% gelatin, for 30 minutes at 37°C. Reverse transcriptase was then inactivated by heating to 95°C for 5 minutes. The $MgCl_2$ concentration was adjusted to 1.5 mM, and 50 pmoles of primer (5'GTCTTCCTCTCTCACGCACCAGC) homologous to rabbit TNF 5' untranslated sequence (8) and 1 unit of Taq DNA polymerase were added. Twenty rounds of amplification were carried out, and the product generated was used as a template for a second round of polymerase chain reaction (pcr), using primers (5'GCGCGGATCCCTGGAGAGAGCGCCATGAGC) and (5'GCGCAAGCTTGGT CAGTCCCCTCACAGGGC) containing Bam HI and Hind III restriction enzyme sites to facilitate cloning and hybridizing immediately upstream and downstream of translated sequences. The DNA fragment generated was cloned into the Bam HI-Hind III sites of vaccinia expression plasmid pMJ601 to generate pMJ601RaTNF.

Human TNF was cloned in a similar fashion, except human U937 cells, stimulated to transcribe TNF with 1 μg/ml LPS for 2 hours, as above, were used as the RNA source, and primers (5' GCGCGGATCCGGACACCATGAGCACT GAAAG) and (5' GCGCAAGCTTGAAGGTTGGATGTTCGTCCTC) from the published human TNF sequence (9) were used to prime reverse transcription and pcr. Human TNF cDNA was cloned as a Bam HI/Hind III fragment into pMJ601 to generate pMJ601HuTNF. Murine TNF was cloned as a Bam HI/Hind III fragment into pMJ601, generating pMJ601MuTNF, from DNA generated by amplification of pMuTNFcDNA (10), obtained from L. Guilbert, using primers (5' GCGCGGATCCGA CACCATGAGCACAGAAAGC) and (5' GCGCAAGCTTACCCATTCCCTTCA CAGAGC). All clones were sequenced to ensure mutations had not been introduced during amplification.

Recombinant vaccinia viruses (strain WR) were constructed as described previously (2) using a Lipofectin^{TM}:DNA (1 μg:1 μg, GibcoBRL) mixture to transfect cells. Recombinant viruses (*tk*$^-$, *lacZ*$^+$) were selected on *tk*$^-$ H143 cells in the presence of 25 μg/ml bromodeoxyuridine and plaque purified three times, using 0.3 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, Bachem) to visualize plaques.

High Level Expression of Rabbit TNF from a Recombinant Vaccinia Virus

The plasmid pMJ601T2RaTNF, used to make a vaccinia virus that overexpresses secreted TNF (VV-T2RaTNF), was constructed in which the rabbit TNF cDNA sequence was placed downstream of the vaccinia synthetic late promoter (7) after the secretory leader sequence of rabbit TNF had been replaced in frame with the secretory leader sequence of the M-T2 protein. The plasmid pUCRaTNF was used as a template for the pcr amplification of the mature rabbit TNF sequence using the primer 5'TGCGTATACGGGCTCAGATCAGCTTCTCGGGCC containing sequence from the M-T2 leader region immediately upstream of sequence encoding the 5' terminal mature rabbit sequence and the M-13 reverse primer 5'AGCGGATAACAATTTCACACAGGA. Similarily, the M-T2 leader sequence containing mature rabbit TNF sequence was amplified using pMTN-6 as template with the primers 5'AGCTGATCTGAG CCCGTATACGCACG CGACCTA and M-13 reverse primer. Twenty rounds of amplification were carried out using 1 unit of Taq DNA polymerase in 50 mM KCl, 10 mM Tris-Cl, pH 8.4, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.25 μM dXTPs, followed by gel purification of the products. The two products with overlapping termini were mixed, denatured by heating to 95^{o}C and allowed to reanneal. Taq DNA polymerase and dXTPs were added to allow 3' extension from the overlapping termini, and a second round of pcr amplification of the full length extension was carried out using M-13 reverse primers. The product was digested with Hind III and cloned into the Hind III site of pBluescript SK- to crease pBlueT2RaTNF. This clone was sequenced to ensure no errors had been introduced during the pcr amplifcation. A Sal I-Bam HI fragment of pBlueT2RaTNF was subcloned into pMJ601 to generate pMJ601T2RaTNF. The recombinant vaccinia virus VV-T2RaTNF (strain WR) was constructed as described above.

Generation of Vaccinia Viruses Expressing MT2 C-Terminal Deletions

The strategy used to generate C-terminal truncations of the M-T2 protein is outlined in Figure II-2. To remove the Sal I restriction endonuclease site present within the multiple cloning site of pMTN-6, pMTN-6 was digested with Xba I and Xho I, the 5' overhangs filled in with the Klenow fragment of DNA polymerase I (GibcoBRL), and religated generating pMTN-6ΔXba/Xho. pMTN-6ΔXba/Xho was then digested with Sal I to generate an exonuclease III sensitive 5' overhang, and Pst I to yield an exonuclease III resistant 3' overhang. The linear DNA was digested with 150 units of exonuclease III in 66 mM Tris-Cl, pH 8.0, 6.6 mM $MgCl_2$, at 37°C. At 30 second intervals, aliquots were removed and treated with 60 units nuclease S1, in 225 mM NaCl, 30 mM KCH_3COO, pH 4.5, 0.2 mM $ZnSO_4$, 4.5% glycerol, at 30°C, for 30 minutes. To generate blunt DNA ends, the nested deletions were first treated with 0.3 units Klenow in 10 mM Tris-Cl, pH 7.6, 200 mM $MgCl_2$, for 15 minutes, at room temperature to remove any remaining 3' overhangs, after which dNTPs were added to 50 mM, and samples incubated a further 15 minutes at room temperature. Xba I linkers ($^{5'}$CTAGTC TAGACTAG) were ligated onto both ends of the linear DNAs, the DNAs were then digested with Xba I and circularized by ligation, generating a series of plasmids (pMTN-ΔL113 to pMTN-ΔD303) with deletions in the C-terminus of the M-T2 open reading frame, followed by an in-frame stop codon. The nested deletion mutants were sequenced to determine the actual site of truncation, and named according to the first amino acid deleted from the sequence of the mature protein.

Hind III/Bam HI fragments, containing the M-T2 C-terminal deletion open reading frames generated above, were cloned into the Hind III/Bam HI sites of the vaccinia virus expression vector plasmid pMJ602 creating the plasmid series pMJ602-MT2ΔL113 to pMJ602-MT2ΔD303. Recombinant vaccinia viruses expressing the M-T2 C-terminal truncations were constructed as described above, and were named according to the first amino acid deleted from the sequence of the M-T2 protein.

Construction of Recombinant Vaccinia Viruses Expressing M-T2 Proteins with Conserved Repeat Deletions

Recombinant vaccinia viruses were constructed to express M-T2 proteins with successive deletions of the four cysteine rich domains (CRDs). The pcr was used to generate the in-frame deletions within the M-T2 coding sequence. To remove the first CRD, pMTN-6 was used as template DNA to amplify sequence upstream of the first repeat, with primers $^{5'}$TGCACGGTCCTCGATCCGCGCCATAC (containing sequence from the non-coding strand immediately downstream and upstream of the first repeat) and M13 reverse primer (M13R) $^{5'}$AGCGGATAACAATTTCACACAGGA in a standard pcr reaction, 1 unit Taq DNA polymerase in 50 mM KCl, 10 mM Tris-Cl, pH 8.4, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.25 μM dXTPs, 20 rounds amplification. To amplify sequence downstream of the first repeat, M13 forward primer (M13F) $^{5'}$GTAAAACGACGGCCACG and primer $^{5'}$TGCACGGTCCTCGATCCGCGCCATAC (containing sequence from the coding strand immediately upstream and downstream of the first repeat) were used in a standard pcr reaction with pMTN-6 as template. Products from the two pcr reactions were gel purified, and the two products with overlapping termini were mixed, denatured by heating to 95°C and allowed to reanneal. Taq DNA polymerase and dXTPs were added for 3' extension from the overlapping termini, followed by a second round of pcr amplification of the full length extension using M13F and M13R as primers. The product was digested with Hind III and Bam HI and subcloned into the Hind III and Bam HI sites of pMJ602 generating pMJ602ΔCR1. The second CRD was deleted in an analogous manner, using primer $^{5'}$AACAGTCAGAACATACCGTGTCGGAC to amplify sequence upstream of the repeat, and primer $^{5'}$GTATGTTCTGACTGTTCTGCCGGG to amplify sequence downstream of the repeat. Primers $^{5'}$GAGGACATTTGCAGACTCTATCGCGGGTT and $^{5'}$AGAGTCTGCAAATGTCCTCGGTACACG were used to delete the third CRD, while primers $^{5'}$CGTTTTAACGTGTGCAGAGCACGTCGCC and $^{5'}$CTCTGCACAGGTTAAAACGTCGGAGTTC were used to delete the fourth CRD. All clones were sequenced to ensure mutations had not been introduced during amplification. The amino acids deleted were as follows: ΔCRD1, K27-S62; ΔCRD2, P63-C104; ΔCRD3, D105-T147; and ΔCRD4, K148-V194. The recombinant vaccinia viruses (strain WR), VV-MT2ΔCR1, VV-MT2ΔCR2, VV-MT2ΔCR3, and VV-MT2ΔCR4 were constructed as described above, using pMJ602ΔCR1 to pMJ602ΔCR4 as vectors for homologous recombination.

Generation of C-terminal M-T2 deletions in Myxoma

Myxoma viruses containing identical C-terminal deletions in each of the two copies of the M-T2 gene were created by homologous recombination with plasmid DNA containing the desired truncations followed by an in frame stop codon, the *E. coli* gpt gene to exert dominant selection pressure and 3' flanking M-T2 sequences. To construct the deletion plasmids, the 218 base pair (bp) Sal I/Pst I fragment, encoding 3' and downstream M-T2 gene sequences was subcloned into the Sal I/Pst I sites of pUC 19, generating plasmid pUC-MT2-3'. The Hind III site within the multiple cloning region was destroyed by digestion with Hind III, flush-end repaired with *E. coli* polymerase I Klenow fragment, and blunt-end ligated. A Hind III site upstream of the Bam HI and M-T2 3' sequence was generated by subcloning in complementary oligonucleotides ($^{5'}$GATCCCCGGGTACCCAAGCTGAGCT and $^{5'}$CAGCTTGGGTACCCGGG) into the Sst I and Bam HI sites. A 940 bp Sal I fragment from p7.5 gptA (3) containing an *E. coli.* gpt cassette downstream of the p7.5 poxviral promoter was subcloned into the Sal I site, oriented with gpt 3' sequence proximal to the M-T2 3' sequence, generating pUCgpt/MT2-3'. The M-T2 C-terminal deletions followed by an in frame stop codon were subcloned from the plasmid series pMTN-ΔL113 to pMTN-ΔD303 as Hind III/Bam HI restriction fragments into the Hind III/Bam HI sites of pUCgpt/MT2-3', yielding the plasmid series, pUCMT2ΔL113 to pUCMT2ΔD303.

Recombinant myxoma viruses were constructed using the pUCMT2Δ plasmids as vectors by infecting BGMK cells with parental vMyxlac virus, and at 5 hours post infection, transfecting cells with a Lipofectin (GibcoBRL):DNA (1µg/1µg) mixture, according to manufacturer's instructions. After 2 days, cells were harvested, and passaged three times with selection medium (25 µg/ml mycophenolic acid, 20 µg/ml xanthine, 15 µg/ml hypoxanthine), and individual recombinants plaque purified three times. The pcr was used to check for correct insertion of DNA into both copies of the M-T2 gene, using appropriate primers flanking the gene.

DETECTION OF M-T2 AND TNF PROTEINS

Generation of Polyclonal Antisera Against Myxoma T2

Antiserum was prepared by intradermally injecting rabbits with purified T2 protein (purification of M-T2 is described subsequently) in Freund's complete adjuvant or with purified T2 protein resolved on a 12% SDS polyacrylamide gel with acrylamide as adjuvant using standard procedures (11). Immunoblots confirmed that the polyclonal antiserum recognized T2 protein from supernatants from myxoma- and VV-MT2- infected cells and did not cross react with any proteins present in supernatants from cells infected with the T2-minus myxoma virus, (vMyxt2g) or control VV-601.

Affinity Purification of M-T2 Antibody

To affinity purify M-T2 antibody present in polyclonal rabbit anti-MT2 antisera, purified M-T2 was resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and visualized by staining with Ponceau-S (Sigma). Nitrocellulose strips containing M-T2 protein were blocked with 10% skim milk in PBS, 0.05% Tween-20, 12 hours, then incubated with 1.5 ml rabbit anti-MT2 antisera, 12 hours, 4^{o}C. Strips were washed with PBS, 0.05% Tween-20, and bound antibody eluted by incubating the strips in 0.5 ml 02 M glycine, pH 2.8, 1 mM EGTA, and then neutralized with 50 µl of 1 M Tris base and50 µl of 10 X PBS.

Detection of TNFs and M-T2 by Western Analysis

Rabbit, murine and human TNFswere detected by Western blotting using a cross-reacting polyclonal rabbit anti-mouse TNF antibody (obtained from L. Guilbert).

Concentrated supernatants from VV-RaTNF, VV-MuTNF or VV-HuTNF infected cells were analyzed by 12% SDS-PAGE, transferred to nitrocellulose in Tris-glycine transfer buffer (48 mM Tris-Cl, pH 9.2, 29 mM glycine, 1.3 mM SDS, 20% methanol) using a semi-dry transfer apparatus (Tyler Industries). The blots were blocked with 10% non-fat skim milk powder, 0.5% Tween-20 in PBS for 2 hours, incubated with a 1:5,000 dilution of anti-mouse TNF antibody for 12 hours followed by a 1:10,000 dilution of horse radish peroxidase- (HRP) conjugated goat anti-rabbit IgG antibody for 2 hours and visualized by ECL chemiluminescence (Amersham) according to the manufacturer's instructions.

Alternatively, to detect M-T2 and rabbit TNF proteins, Western blots were probed with a 1:5000 dilution of rabbit anti-T2 antisera, a 1:1000 dilution of affinity purified anti-MT2 antibody, or a 1:2000 dilution of a goat anti-rabbit TNF antibody (Pharmingen) as indicated, followed by the appropriate secondary antibody diluted 1:5000 for HRP-conjugated goat anti-rabbit IgG antibody or 1:2000 for HRP-conjugated donkey anti-goat IgG antibody, and immunoblots were visualized by ECL chemiluminescence.

Radiolabelling of T2 Protein Expressed from Vaccinia Infected Cells

To detect myxoma T2 protein secreted from VV-MT2 infected cells, 80% confluent BGMK monolayers in 6-well multidishes were adsorbed with virus in a volume of 100 μl at a multiplicity of infection (m.o.i.) of 10. After 1 hour, DMEM with 10% NCS was added and the infection allowed to proceed for varying lengths of time. Two hours prior to harvest, the cells were washed twice with PBS, and then incubated 1 hour in 0.4 ml serum-free, Met-free DMEM containing 100 μCi ^{35}S Translabel ([^{35}S]-Met/Cys mixture; ICN Biomedicals). The media was removed, and the cells incubated 1 hour in 0.25 ml serum-free DMEM. The supernatant was collected, and the protein precipitated with 1 ml methanol:acetone (4:1) and pelleted in a benchtop microfuge for 10 minutes. The pellet was resuspended in 50 μl Laemmli sample buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and 20 μl was loaded onto a 10% SDS-polyacrylamide gel. The gel was dried onto Whatman 3MM chromatography paper and analyzed by autoradiography.

Immunoprecipitation of Intracellular and Secreted M-T2 Protein

BGMK cells (10^6 cells/well of a 6-well dish) were infected with VV-MT2, VV-601, vMyxlac or vMyxt2g at a m.o.i. of 10. At the designated time, cells were washed with PBS and incubated in 0.5 ml serum-free, Met-free, Cys-free DMEM containing 100 µCi [^{35}S] Express Protein Label ([^{35}S]Met/Cys mixture, Dupont NEN) for the time indicated, after which the medium containing secreted, radiolabeled M-T2 was collected, the cells washed once in PBS, and lysed with NP40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-Cl, pH 8.0) containing 0.05% vol/vol SDS. Supernatants and lysates were precleared by incubation with Protein A beads (Protein A Sepharose CL-4B, Pharmacia, 30% vol/vol in NP40 lysis buffer) for 1 hour at 4°C, after which the beads were pelleted by centrifugation at 10,000 x g for 1 min. To immunoprecipitate M-T2 protein, 1 µl of affinity purified rabbit anti-MT2 antiserum was added, the mixture incubated for 1 hour at 4°C, followed by the addition of 30 µl of Protein A beads. The antibody-antigen reaction was incubated a further 30 min. at 4°C, after which the beads were collected by centrifugation at 10,000 g for 1 min., and the immune complexes washed three times with NP-40 lysis buffer, resuspended in 50 µl of Laemmli sample buffer and resolved by SDS-PAGE. Gels were fixed 1 hour in 5% acetic acid, 15% methanol, impregnated with EN^{3}HANCE (Dupont), dried and analyzed by fluorography.

Visualization of Vaccinia Virus Expressed M-T2 Deletion Protein

BGMK cells were infected at a m.o.i. of 10 with VV-601, VV-MT2 or the different VV-MT2ΔC-terminus vaccinia viruses, and after 24 hours, the supernatants were harvested, the cells were washed with PBS, and lysed with 200 µl Laemmli sample buffer. Protein present in supernatants was precipitated with 4 volumes of methanol:acetone, 1:1 vol/vol, pelleted by centrifugation at 10,000 x g, 10 min., and resuspended in Laemmli sample buffer. Intracellular protein from 5 x 10^4 infected cells and secreted protein from 5 x 10^5 VV-601 and VV-MT2ΔC-terminus-infected cells and 5 x 10^4 VV-MT2 infected cells, were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized by Western analysis as above, probing with 1:1000 dilution affinity purified anti-MT2 antibody.

To observe the M-T2 CRD deletion proteins, 10^6 BGMK cells were infected at a m.o.i. of 10 with the different vaccinia viruses expressing the M-T2 CRD deletions, VV-MT2ΔCRDs. Six hours post-infection, the cells were washed with PBS, and incubated 16 hours in 0.5 ml serum-free, Met-free, Cys-free DMEM containing 100 μCi [^{35}S] Express Protein Label ([^{35}S]Met/Cys mixture, Dupont NEN). Supernatants were then collected, and the cells washed once in PBS and lysed with 200 μl NP-40 lysis buffer. M-T2 CRD deletions present in supernatants and cell lysates were immunoprecipitated as above, washing the Protein A-antibody complexes with NP-40 lysis buffer in the absence of SDS, and immunoprecipitated proteins were resolved by SDS-PAGE and visualized by fluorography.

VIRUS REPLICATION IN TISSUE CULTURE

Replication of Viruses in Tissue Culture

Duplicate wells of semiconfluent monolayers of BGMK, RK-13, SIRC and L929-8 cells in 24-well multidishes were adsorbed with vaccinia virus expressing the different TNFs, at a m.o.i. of 0.1, 0.01 or 0.001 in a volume of 100 μl for 1 hour at 37°C. Then, 0.5 ml of medium was added to each well, and the cells were incubated a further 48 hours at 37°C. Cells were removed with 0.15 M NaCl, 0.015 M sodium citrate, pelleted, and resuspended in 200 μl 10 mM Tris-Cl, pH 8.0, 2 mM $MgCl_2$. Virus was released from cells with three freeze/thaw cycles, and virus titers were determined by plaque assay on BGMK cells and staining of fixed monolayers with X-gal as described (2).

To determine the effect of rabbit TNF on virus growth in rabbit RK-13 cells, semiconfluent monolayers of RK-13 cells in 6-well multidishes were adsorbed with 50 plaque forming units (p.f.u.) virus (VV-601, VV-MT2, vMyxlac, vMyxt2g) in quadruplicate for 1 hour in a volume of 200 µl. Six hours post-infection, 0, 1, 2 or 10 ng/ml rabbit TNF was added. Cells were harvested 72 hours post-infection, and virus titers determined by plaque assay, as described above.

Co-infection of cells with VV-RaTNF and VV-MT2

L929-8 cells (10^6 cells/well in 6-well dishes) were co-infected in triplicate with VV-MT2 and VV-RaTNF of VV-601 and VV-RaTNF, 9 p.f.u.:1 p.f.u. at a m.o.i. of 10 for 24 hours, or 1 p.f.u.:1 p.f.u. at a m.o.i. of 0.2 for 48 hours. Cells were then harvested and the virus titers determined by plaque assay as described above.

Effect of rabbit TNF on the Replication of Myxoma Knock-Out Viruses

RK-13 cells (10^6 cells/well in 6-well dishes) were infected in triplicate with vMyxlac, vMyxt2g, vMyxM11L$^-$ or vMyxSERP$^-$ at a m.o.i. of 0.1, in the presence or absence of 1 nM rabbit TNF. After 48 hours, cells were harvested and the virus titers determined by plaque assay as described above.

Replication of Myxoma M-T2 C-Terminal Deletion Viruses

10^6 BGMK or RL-5 cells were adsorbed with vMyxlac, vMyxt2g (M-T2-knockout), myxoma (strain Lausanne), vMyxt2r (M-T2-revertant), or the different myxoma viruses expressing M-T2 C-terminal truncations at a m.o.i. of 1, 1 hour at 37°C. Cells were then washed once with PBS, and incubated in medium 36 hours at 37°C, after which the cells were removed with 0.15 M NaCl, 0.015 M sodium citrate, pelleted, and resuspended in 200 µl 10 mM Tris-Cl, pH 8.0, 2 mM $MgCl_2$. Virus was released from cells by three freeze/thaw cycles, and virus titers were determined by plaque assay on BGMK cells following staining of fixed monolayers with X-gal or 0.1 % crystal violet, as described (2). To determine the effect of rabbit TNF on virus growth, RL-5 cells were infected with vMyxlac or vMyxt2g in the presence of 50 nM rabbit TNF, and at the times indicated, the cells harvested and virus titres measured as described above.

KINETICS OF M-T2 PRODUCTION AND SECRETION

Quantitation of M-T2 Protein Secreted from Virally-Infected Cells

Western analysis was used to estimate the amount of M-T2 protein secreted from from vMyxlac and VV-MT2 infected cells, probing with a 1:5000 dilution of rabbit anti-M-T2 antiserum followed by a 1:5000 dilution of HRP-conjugated goat anti-rabbit IgG antibody (Biorad Laboratories). The intensities of the bands visualized were measured by scanning using a Joyce Loebl chromoscan 3 densitometer. The results of four separate Western blots were averaged to determine the amount of M-T2 protein present in supernatants, using various titrations of purified M-T2 protein as the standard.

Time Course of M-T2 Synthesis and Secretion from Myxoma-Infected cells

BGMK cells (10^6 cells/well of a 6-well dish) or RL-5 cells (2×10^6 cells) were infected with vMyxlac or vMyxt2g at a m.o.i. of 20. At the times indicated, cells were washed with phosphate buffered saline (PBS) and incubated in 0.5 ml serum-free, Met-free, Cys-free DMEM containing 100 μCi ^{35}S Express Protein Label ([^{35}S]Met/Cys mixture, Dupont NEN) as indicated, after which the medium containing secreted, radiolabeled M-T2 was collected, the cells washed once in PBS, and lysed with NP40 lysis buffer containing 0.05% vol/vol SDS. Supernatants and lysates were precleared by incubation with Protein A beads, after which the M-T2 protein was immunoprecipitated with 1 μl of affinity purified rabbit anti-MT2 antibody and visualized as described above. To immunoprecipitate M-T7, supernatants from infected cells were incubated with 1 μl affinity purified rabbit anti-MT7 antibody (12), and the M-T7 protein immunoprecipitated as above.

Pulse Chase Analysis of Intracellular and Secreted M-T2 Protein

BGMK cells (10^6) or RL-5 cells (2×10^6) were infected with virus, vMyxlac or vMyxt2g, at a m.o.i. of 10. Thirty minutes prior to the labelling time, cells were washed with PBS and incubated in 0.5 ml serum-free, Met-free, Cys-free DMEM. Cells were then incubated in 0.5 ml serum-free, Met-free, Cys-free DMEM containing 100 µCi ^{35}S Express Protein Label ([^{35}S]Met/Cys mixture), after which they were washed with PBS and incubated in DMEM or RPMI for increasing amounts of time. Supernatants and cell lysates were harvested, M-T2 protein immunoprecipitated and resolved by SDS-PAGE as described above.

Northern Blotting of M-T2 mRNA

BGMK cells were infected with vMyxlac or vMyxt2g at a m.o.i. of 20, and RNA from infected cells extracted at various time points post-infection using TRIzol Reagent (Life Technologies). Early RNA was prepared from infected cells cultured in the presence of 40 µl/ml arabinosyl cytosine (araC). Cells were lyzed with TRIzol reagent (1.0 ml/10^6 cells), 0.2 ml chloroform was added, and samples centrifuged 12,000 x g, 15 min., 4°C. The aqueous phase, containing RNA separated from cellular DNA and proteins, was collected and the RNA was precipitated with 0.5 ml isopropanol, pelleted by centrifugation 12,000 x g, 10 min., and redissolved in 0.5% SDS. RNA samples (3 µg) were denatured by incubating at 55°C for 15 minutes in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) containing 50% deionized formamide and 20% formaldehyde, and resolved by electrophoresis through a 1% agarose gel containing 1 X MOPS buffer, 2% formaldehyde, 0.5 µg/ml ethidium bromide, and transferred to nitrocellulose by capillary action. Nitrocellulose membranes were baked in a 80°C oven for 2 hours, prehybridized and hybridized in 50% formamide, 0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 2X Denhardt's solution, 0.1% SDS. For hybridization, an M-T2 probe was prepared by random priming using a 0.9 kb M-T2 pcr product with [α-^{32}P]dCTP (DupontNEN), and random primer (Boehringer Mannheim). Blots were washed in 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS at 55°C, and bound probe visualized by autoradiography.

PURIFICATION OF M-T2 and RABBIT TNF AND BIOCHEMICAL CHARACTERIZATION OF M-T2

Preparation of Concentrated Supernatants from Virus-Infected Cells

Roller bottles of BGMK cells (3 x 10^8 cells/bottle) were adsorbed with virus at an m.o.i. of 1 in a volume of 10 ml serum-free DMEM at 37 °C for 4 hours. Cells were rinsed three times with PBS, and incubated a further 18-24 hours in 25 ml serum-free DMEM. The media was collected, centrifuged 15,000 x g for 30 minutes, concentrated 20-fold using Centriprep-10 filters (Amicon) or an ultrafiltration cell (Amicon), and then centrifuged for 2 hours at 50,000 xg.

To determine the TNF levels present in the concentrated supernatants, aliquots were run out on a 15% polyacrylamide gel and visualized by silver staining (13). The gel was dried between two sheets of biogel wrap, and scanned using a Joyce Loebl chomoscan3 densitometer. Using purified recombinant human TNF (C. Smith, Immunex Corp.) as a standard, the intensity of bands determined to be TNF by Western blotting were used to calculate the molar concentrations of each TNF sample.

Purification of Myxoma T2 Protein and Rabbit TNF

Myxoma T2 protein was purified from media from VV-MT2-infected BGMK cells in roller bottles (3 x 10^8 cells). Supernatants were prepared as described above, and the media from 8-16 roller bottles was concentrated using an Amicon ultrafiltration cell to a volume of 20-30 mls and dialyzed overnight against 20 mM Tris-Cl, pH 7.5 at 4°C. The dialyzed sample was applied to a Mono-Q anion exchange column (Pharmacia) and protein was eluted using a linear salt gradient, 0-500 mM NaCl in 20 mM Tris-Cl, pH 7.5. Fractions containing M-T2 were pooled, dialyzed against 20 mM bis-Tris pH 6.4, and re-applied to a Mono-Q anion exchange column. Protein was eluted using a 0-500 mM NaCl salt gradient at pH 6.4. M-T2-containing fractions were analyzed for purity by silver staining of polyacrylamide gels (13).

Rabbit TNF was purified from supernatants from VV-T2RaTNF infected BGMK cells in roller bottles in an identical fashion, using pH 7.5 Mono-Q anion-exchange chromatography followed by pH 6.4 anion-exchange chromatography. The M-T2 or rabbit TNF protein present in the different fractions was confirmed by silver staining and quantitated by O.D.$_{280}$ measurements.

Gel Filtration Chromatography of Purified Myxoma T2 Protein

A Superdex 200 gel filtration column (Pharmacia) was calibrated using thryoglobulin, ferritin, catalase, aldolase and bovine serum albumin (Pharmacia) as size standards. Purified T2 protein was loaded onto the calibrated Sephadex 200 gel filtration column equilibrated with PBS and fractions collected. Fractions containing protein were resolved by SDS-PAGE under reducing (50 mM Tris-Cl, pH 6.8, 100 mM dithiotreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol sample loading buffer) or non-reducing conditions (50 mM Tris-Cl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol sample loading buffer), transferred to nitrocellulose and probed with 1:5000 dilution of rabbit anti-M-T2 antisera as above.

Sedimentation Equilibrium Analysis

Sedimentation equilibrium studies were performed on a Beckman Spinco Model E analytical ultracentrifuge using absorbance optics. The run was performed at 8000 revolutions per minute in PBS at 20^{o}C. The molecular weight (M) was calculated using the equation:

$$M = \frac{2RT}{(1 - v\rho)\omega^2}\left[\frac{d(\ln C)}{d(r^2)}\right]$$

where R is the universal gas constant, T is the temperature in Kelvin, ∇ is the partial specific volume, ρ is the solvent density, ω is the angular velocity, and $d(\ln C)/dr^2$ is the slope from the plot in Figure IV-5. Based on the amino acid composition of M-T2 protein, ∇ was calculated to be 0.69. As M-T2 is glycosylated, ∇ for the glycoprotein was calculated to be 0.66 using the formula $(M_1\nabla_1 + M_2\nabla_2)/(M_1 + M_2)$, where M_1, ∇_1

and M_2, $\bar{v}_2$ are the masses and partial specific volume of the protein and the sugar component respectively.

Peptide-N-glycosidase F Digestion of Myxoma T2 Protein and Lectin Overlays

Purified T2 protein, 1 µg in 100 mM sodium phosphate, pH 7.0, 1% SDS, was boiled for 10 minutes to denature the protein, and diluted 10-fold with 100 mM sodium phosphate, pH 7.0, 2.0 mM EDTA, 1% TritonX-100, 1 mM dithiothreitol. One unit of peptide-N-glycosidase F (Boehringer Mannhein) was added and the reaction carried out overnight at 37°C. Reaction products were analyzed by 12% SDS-PAGE, immunoblotted and probed with 1:5000 dilution rabbit anti-T2 antisera followed by a 1:5000 dilution of HRP-conjugated goat anti-rabbit IgG antibody and visualized by ECL chemiluminescence (Amersham).

Lectin overlays to measure M-T2 binding to concavalin A (Con A) or *Ricinus communis* agglutinin (RCA) were performed as described in detail by Bar-Nun and Gershoni (14). Briefly, N-glycosidase digested and undigested M-T2 protein was resolved by SDS-PAGE, transferred to nitrocellulose, and the membrane blocked overnight with 2% bovine serum albumin (BSA) in 25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.5% Tween-20. The membrane was probed 1 hour with 10 µg/ml biotin-conjugates of Con A or RCA (Sigma) in 25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.5% Tween-20, and 1 mM $CaCl_2$ for Con A overlays only. Bound lectin was visualized by probing with 1:2000 dilution of avidin-HRP conjugate (Biorad) followed by ECL chemiluminescence (Amersham).

Endo-H Analysis of Intracellular and Secreted M-T2 Protein

Protein A beads containing immunoprecipitated M-T2 protein or M-T7 protein were washed with PBS and divided into two aliquots. Protein was denatured by boiling samples 10 minutes in 20 µl 0.5% SDS, 1% β-mercaptoethanol, after which 2 µl of 500 mM sodium citrate, pH 5.5 was added, and to one of the aliquots, 1000 units of Endo H_f (New England BioLabs). Samples were incubated 1 hour at 37°C, and reaction products analyzed by SDS-PAGE, followed by fluorography or Western analysis, as described above.

BINDING OF M-T2 PROTEIN AND TNF

Co-immunoprecipitation of M-T2 and Rabbit TNF

Supernatants or lysates containing ^{35}S-labelled M-T2 or rabbit TNF were prepared from vMyxlac-, VV-MT2 or VV-T2RaTNF-infected cells, as described above, labelling cells with 100 µCi [^{35}S] Express Protein Label from 4-20 hours post infection, and the supernatants and lysates collected were precleared with Protein A beads. 100 µl supernatants from VV-MT2, VV-T2RaTNF or VV-601-infected cells were coincubated for 1 hour at 4°C, after which 1 µg of purified goat anti-rabbit TNF-α antibody (PharMingen) or 1:200 dilution of rabbit anti-T2 antiserum was added and the mixture incubated for 1 hour further at 4°C. Next, 30 µl of Protein A beads (10% vol/vol in NP40 lysis buffer) was added, and the antibody-antigen reaction incubated 1 hour further at 4°C. The beads were collected by centrifugation at 10,000g for 1 minute at 4°C, and the immune complexes were washed three times with NP-40 lysis buffer, resuspended in 50 µl of Laemmli sample buffer, resolved by SDS-PAGE and analyzed as described above. Alternatively, 25 ng of purified T2 protein was incubated wtih 100 µl of [^{35}S]-met/cys labeled supernatant from VV-T2RaTNF-infected cells, and coimmunoprecipitations were performed as above using a 1:200 dilution of rabbit anti-M-T2 antiserum.

To co-immunoprecipitate rabbit TNF with M-T2 deletion mutants, BGMK cells were infected with the various VV-MT2ΔC-terminus or VV-MT2ΔCRD viruses at a m.o.i. of 10; after 24 hours, supernatants were collected, the cells were washed with PBS, and lysed with NP-40 lysis buffer. Supernatants from the equivalent of 2 x 10^5 VV-T2RaTNF-infected cells, containing [^{35}S]-met/cys-labeled RaTNF labelled as described above, were combined with supernatants from 2 x 10^5 VV-MT2 infected cells, or 10^7 VV-MT2ΔC-terminus- or VV-MT2ΔCRD-infected cells, or with lysates from 5 x 10^5 infected cells, and incubated 30 min. at 4°C, and M-T2 protein complexes co-immunoprecipiated with 1 µl of affinity purified rabbit anti-T2 antibody as described above. Alternatively, rabbit TNF was immunoprecipitated from supernatants from [^{35}S] met/cys-labeled, VV-T2Ra-infected cells with 1 µg of purified goat anti-rabbit TNF antibody (Pharmingen), and collected on Protein A beads as above.

To co-immunoprecipitate the M-T2 truncations expressed in myxoma virus with rabbit TNF, BGMK cells (10^6 cells) were infected with vMyxlac, vMyxt2g, or the different viruses expressing the various M-T2 mutant proteins at a m.o.i. of 10 and after 24 hours, supernatants were harvested and cells lysed with NP-40 lysis buffer. Supernatants from the equivalent of 2 x 10^5 VV-T2RaTNF-infected cells, containing [^{35}S] met/cys-labeled RaTNF labelled as described above, were co-incubated for 30 min. at 4°C with total cell protein from 10^6 infected cells, and immune complexes immunoprecipitated with 1 µl of affinity purified rabbit anti-T2 antiserum as above.

Solid Phase M-T2 Binding Assay

Purified rabbit TNF, human TNF (Quality Controlled Biochemicals) and mouse TNF (PharMingen) were labeled with Na^{125}I (Dupont NEN) to a specific activity of 10^5 Ci/mole using lactoperoxidase and hydrogen peroxide according to standard procedures (11). Falcon 96-well immunoplates were coated with 100 ng of purified M-T2 protein in 50 µl of PBS, overnight at 4°C. Wells were blocked with 5% skim milk powder in PBS for two hours at room temperature, followed by binding of ^{125}I-labeled TNF in 50 µl blocking buffer for 2 hours. Wells were washed three times with PBS, removed and counted on a Beckman Gamma 5500 Radiation Counter. To determine the specific binding of M-T2 to TNF, non-specific binding in the presence of 100-fold excess cold TNF was subtracted from total binding, and such non-specific binding routinely represented less than 5% of total binding. All assays were performed in triplicate, and binding of ^{125}I-labeled TNF to myxoma T2 was analyzed by the method of Scatchard (15).

M-T2 Inhibition of Binding of ^{125}I-Labeled Rabbit TNF to RK-13 and RL-5 Cells

To measure specific binding of ^{125}I-labeled rabbit TNF to RL-5 (rabbit T cell lymphoma) cells, 2 x 10^6 cells were seeded in microfuge tubes in 100 µl of RPMI medium supplemented with 10% FBS, 500 pM ^{125}I-labeled rabbit TNF, in the presence or absence of 100-fold excess cold TNF, for 1 hour at 4°C. The cell suspension was then layered over 250 µl transport oil (15% paraffin oil, 85% silicon 550 oil, specific gravity 1.032 g/ml) in microfuge tubes, the cells pelleted through the oil by centrifugation at 10,000 g, 1 min, and the oil and cell pellet washed three times with water. The cell pellet was resuspended in 0.5% SDS and the radioactivity present in the cell pellet measured on a Beckman 5500 Gamma Counter. VV-601 and VV-MT2 supernatants were prepared as described above. To test the ability of M-T2 protein to inhibit binding, 2 x 10^6 cells were incubated with 500 pM ^{125}I-labeled rabbit TNF and 200 nM purified M-T2 protein or 10% vol/vol VV-601 supernatant or VV-MT2 supernatant containing 50 nM M-T2 protein, and processed as described above. All assays were performed in triplicate.

To measure TNF-binding to RK-13 (rabbit fibroblast) cells, 1 x 10^6 RK-13 cells seeded in 6-well dishes were incubated in 200 µl DMEM supplemented with 10% NCS. ^{125}I-labeled rabbit TNF was added to a concentration of 500 pM, in the presence or absence of 100-fold excess cold TNF to measure specific binding, or in the presence of 20 nM purified M-T2 protein, and cells were incubated 1 hour at 4°C with gentle agitation. Cells were then washed three times with PBS, resuspended in 200 µl 0.5% SDS, and the radioactivity remaining in the sample measured on a Beckman 5500 Gamma Counter. All assays were performed in triplicate.

ASSAYS OF CYTOLYSIS, GROWTH INHIBITION AND APOPTOSIS

TNF-Induced Cytolysis of Actinomycin D-Sensitized Cells

For TNF cytolysis assays, cells were plated out in 96-well dishes at a density of 5 x 10^4 cells/well for L929-8 cells, or 2 x 10^4 cells/well for all other cell lines, in the presence of 2 μg/ml actinomycin D. The cells were incubated 2 hours at 37°C, TNF was added and the cells incubated a further 18-20 hours at 37°C. Cell viability was measured using the vital stain neutral red, as described (5) with the following modifications. One hundred μl of 0.05% neutral red (w/v) in PBS was added to each well and cells were incubated 2 1/2 hours at 37 °C. The cells were then washed with PBS, and 100 μl of 50% ethanol (v/v), 0.05M NaH_2PO_4 was added to each well. Absorbance at 570 nm was measured using a Dynatech MR600 automated microplate sphectrophotometer with a reference wavelength of 630 nm. Medium minus TNF was used as a control to monitor cell viability, and optimized concentrations of TNF were used as a positive control for maximum cytolysis. Results are expressed as % viability, the mean of quadruplicate samples by the formula $\frac{OD_{\text{experimental}} - OD_{\text{maximum killing}}}{OD_{\text{maximum viability}} - OD_{\text{maximum killing}}} x100$. To detect TNF secreted from rabbit cells, supernatants from rabbit peripheral blood lymphocytes were prepared as described above. Alternatively, supernatants from RL-5 cells, RL-5 cells treated with 1 μg/ml LPS (Sigma) for 12 hours, or vMyxlac-infected or vMyxt2g-infected RL-5 cells, infected for 12 hours at a m.o.i. of 10, were collected and concentrated 10-fold with Ultrafree-MC 10,000 NMWL filter units (Millipore). The presence of TNF was measured by the induction of cytolysis in actinomycin-D L929-8 cells and measuring the uptake of neutral red by viable cells as described above.

T2-Protection Against TNF-Induced Cytolysis

For protection experiments to assess the ability of myxoma T2 to inhibit TNF cytolysis, cells were pre-incubated as described above in the presence of 2 μg/ml actinomycin D, before adding simultaneously either rabbit, human or mouse TNF and varying molar ratios of excess T2. TNF was used at concentrations in which cell killing was >95% for L929-8 cells and >65% for RK-13 cells. T2 protein was added at various molar ratios of T2:TNF calculated based on the mass of each monomer, unless stated otherwise. Cell viability was determined by neutral red uptake as above for L929-8 cells, and by crystal violet staining for RK-13 cells, as described (16). The ability of goat anti-rabbit TNF antibody (Pharmingen) to inhibit the cytolysis of L929-8 cells by rabbit TNF was determined by incubating actinomycin-D sensitized L929-8 cells with various concentrations of rabbit TNF and either 2.5 µg/ml goat anti-rabbit TNF antibody, for 18 hours at 37°C and the cell viability was measured by neutral red uptake. L929-8 cells incubated with medium alone were used as a reference for maximum viability, and L929-8 cells incubated in the presence of 100 nM mouse TNF were used as a reference for maximum cytolysis. Results are expressed as % viability, the mean of triplicate samples by the formula: $\frac{OD_{experimental} - OD_{maximum\ killing}}{OD_{maximum\ viability} - OD_{maximum\ killing}} x100$, except when the results are expressed as % increase in viability, in which case the formula (% viability experimental - % viability in the absence of M-T2) was used.

When the ability of myxoma T2 protein, and the various M-T2 deletion mutants to protect mouse L929-8 cells from rabbit TNF-induced cytolysis was measured, the amount of M-T2 deletion protein present in supernatants from cells infected with the vaccinia viruses expressing the various M-T2 deletion mutants was estimated from the intensity of the signal observed on Western blots. Supernatants containing approximately 500 ng/mL M-T2 deletion protein for deletion mutants ΔN169-ΔD303 and ΔCRD1-ΔCRD3, 100 ng/ml ΔL113, 500 ng/ml (12.5 nM) purified M-T2 protein, or 10% (vol/vol) supernatant from control VV-601-infected were tested for their ability to inhibit cytolysis by rabbit TNF, and after 18 hours, cell viability was determined by neutral red uptake (5). Results are expressed as the % of viable cells compared to untreated control cells, the mean of quadruplicate samples calculated by the formula $\frac{OD_{experimental} - OD_{maximum\ killing}}{OD_{maximum\ viability} - OD_{maximum\ killing}} x100$.

Growth Inhibition by TNF

For TNF growth inhibition ("cytostatic") assays, RK-13 or SIRC cells were plated out in 96 well dishes at a density of 10^4 cells/well, and incubated at 37°C, at various TNF concentrations, for up to 5 days before staining with neutral red, as above. Results are expressed as the % of viable cells compared to untreated control cells, the mean of quadruplicate samples calculated by the formula above.

Growth of RL-5 Cells in the Presence and Absence of Rabbit TNF

RL-5 cells (10^5 cells in 0.5 mL) were plated out in quadruplicate in 24 well-dishes in the presence and absence of 50 nM rabbit TNF. At the times indicated, cells were removed with 0.15 M NaCl, 0.015 M sodium citrate, and counted on a hemocytometer.

Sensitivity of L929-8 Cells to Cytolysis by RL-5 Cells

L929-8 cells were plated out in 96-well dishes at a density of 5 x 10^4 cells/well in the presence of 2 µg/ml actinomycin D, and the cells incubated at 37°C for 2 hours. The cells were then overlayed with various dilutions of RL-5 cells (7.5 x 10^5 - 5 x 10^3 cells/well) in the presence or absence of 100 nM purified M-T2 protein, or 2.5 µg/ml goat anti-rabbit TNF antibody (Pharmingen), or overlayed with RL-5 cells previously infected with vMyxlac or vMyxt2g for 12 hours at a m.o.i. of 10. After 18 hours, 100 µl of 0.05% neutral red (w/v) in PBS was added to each well and the cells were incubated 2 1/2 hours further at 37°C. Cells were then washed once with PBS, and neutral red uptake by viable cells measured, as described above. The ability of M-T2 protein or goat anti-rabbit TNF antibody (Pharmingen) to inhibit the cytolysis of L929-8 cells by rabbit TNF was determined by incubating actinomycin-D sensitized L929-8 cells with various concentrations of rabbit TNF and either 2.5 µg/ml goat anti-rabbit TNF antibody or 2%

RPMI medium containing 500 nM purified M-T2 protein, for 18 hours at 37°C, and the cell viability was measured by neutral red uptake, as described above. L929-8 cells incubated with medium alone were used as a reference for maximum viability, and L929-8 cells incubated in the presence of 100 nM mouse TNF were used as a reference for maximum cytolysis.

Quantitation of Apoptotic Cells by Flow Cytometry

RL-5 cells (2 x 10^6 cells) were mock-infected, or infected with virus at a m.o.i. of 10 for 10 hours, after which cells were harvested for TUNEL staining. Where indicated, infections were carried out in the presence of 500 nM purified M-T2 protein. The proportion of cells showing DNA fragmentation was measured by incorporation of fluorescein-12-dUTP (FITC-12-dUTP, Boehringer Mannheim) into DNA using terminal deoxynucleotidyl transferase (TdT; Boehringer Mannheim) and quantitation by flow cytometry as described(17). Briefly, cells were washed in PBS containing 1% serum, fixed in 2% paraformaldehyde in PBS for 30 min. at room temperature, washed once with PBS containing 1% serum, and permeabilized with 0.1% Triton X-100, 0.1% sodium citrate, on ice for 2 min. The permeabilized cells were washed twice with PBS containing 1% serum, and incubated in TdT reaction mixture (6 µM FITC-12-dUTP, 60 µM dATP, 1 mM $CoCl_2$, 25 U TdT, in 30 mM Tris-Cl, pH 7.2, 140 mM sodium cacodylate). Non-specific uptake of label was measured by reacting the cells in the absence of TdT. Reactions were stopped in 20 mM EDTA, and the cells were subjected to flow cytometric analysis on a FACScan (Becton Dickinson) using a 488 nM argon ion laser to excite fluorescent emmissions detected through a 530 nm band pass filter. For each sample 10000 events were collected.

Figure II-1. Structure of Recombinant Viruses

Myxoma T2, rabbit TNF, human TNF and mouse TNF coding sequences were placed under the control of a vaccinia synthetic late promoter (SLP), and inserted into the *th*ymidine kinase, *tk*, locus of vaccinia (WR). The vector control virus, VV-601 contains only lacZ inserted into *tk*. sequences (▩); N-terminal leader sequence (■ for M-T2 and ▧ for TNF), putative cleavage sites (↑), TNF coding sequence (▦), cysteine-rich repeats (□), cysteine-rich domains (⬭) cysteine residues (|), and potential N-linked glycosylation sites (*) are shown. VV-T2RaTNF contains the M-T2 leader preceding the coding sequence of mature rabbit TNF. The various vaccinia viruses and myxoma viruses expressing C-terminal truncations or internal deletions are aligned with the sequence of full-length M-T2, as indicated.

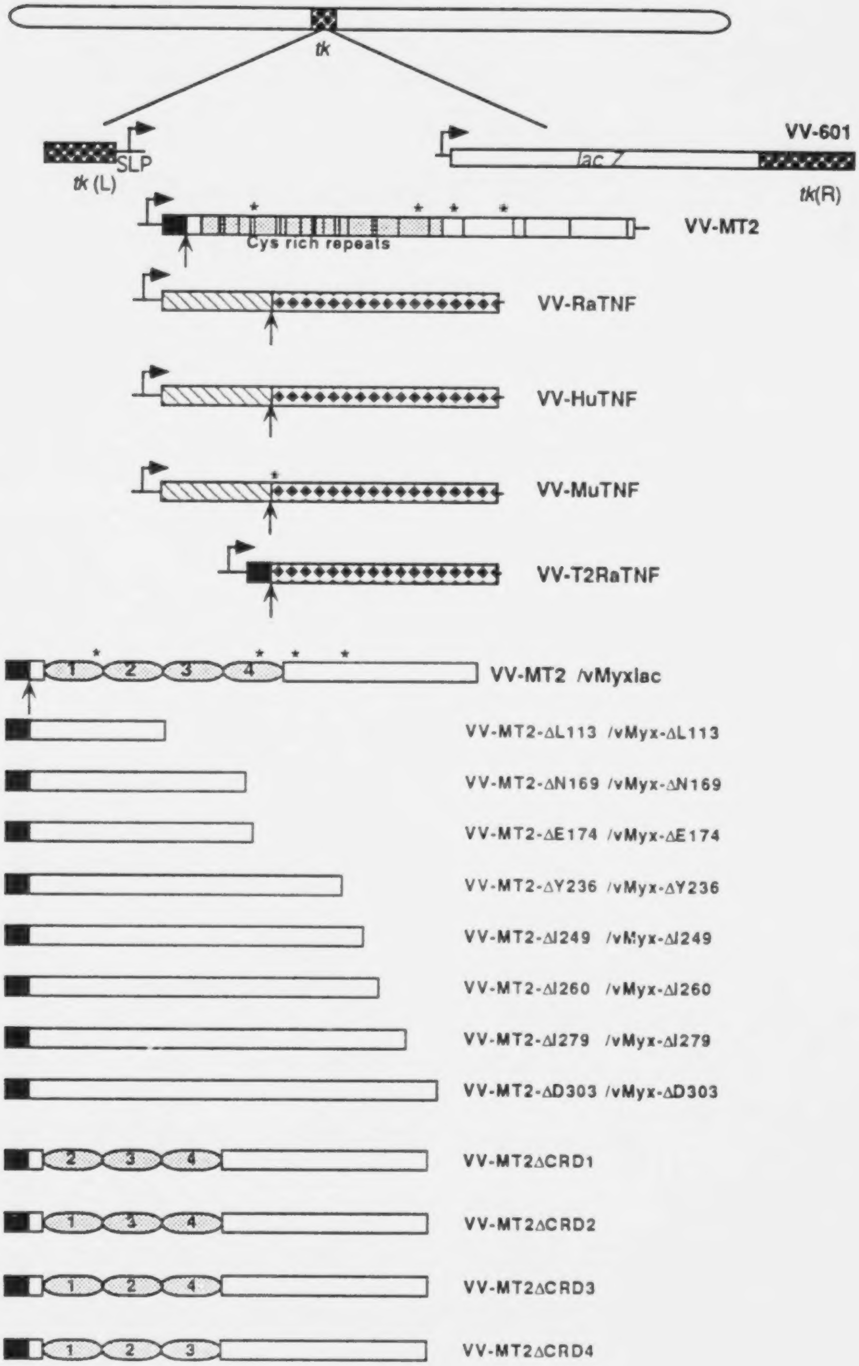
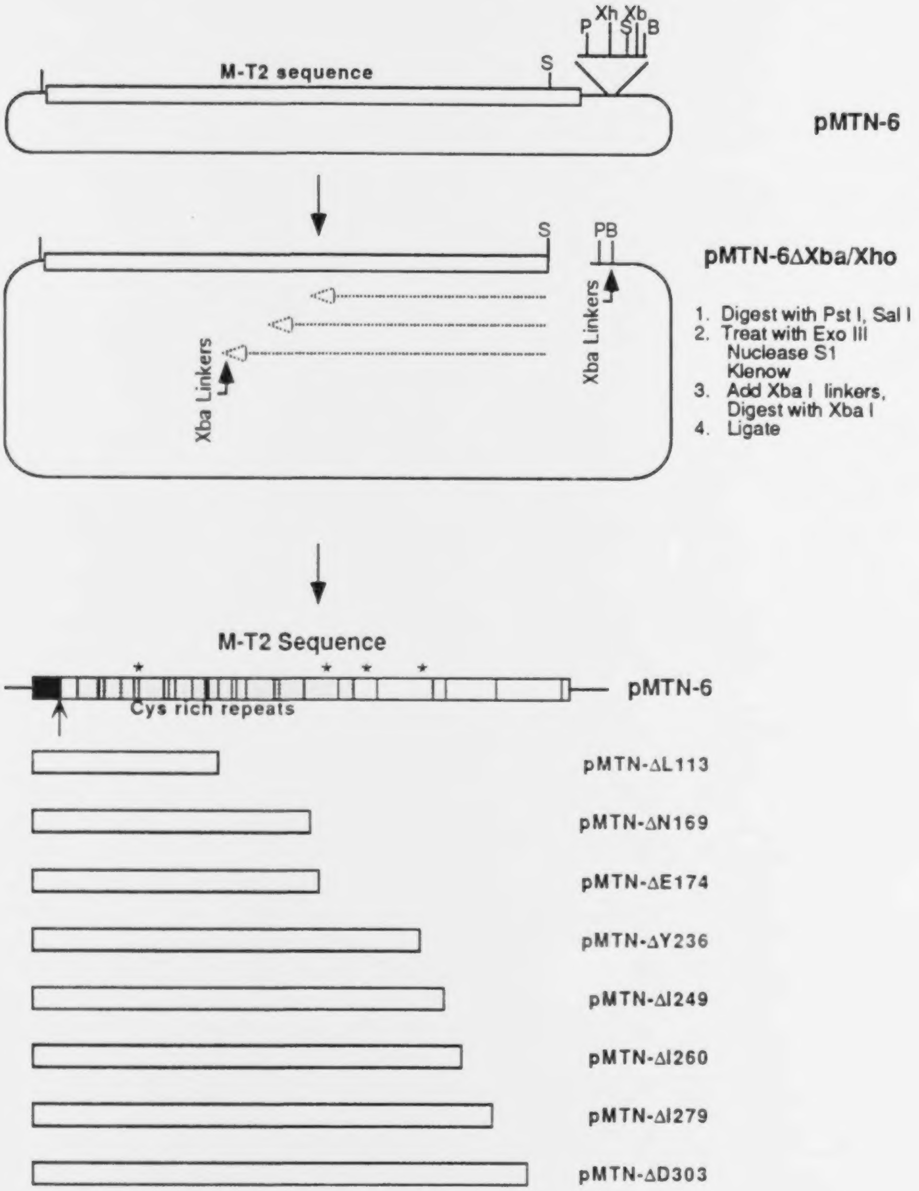

tk
VV-601
SLP
tk (L)
lac Z
tk(R)
Cys rich repeats
VV-MT2
VV-RaTNF
VV-HuTNF
VV-MuTNF
VV-T2RaTNF
1
2
3
4
VV-MT2 /vMyxlac
VV-MT2-ΔL113 /vMyx-ΔL113
VV-MT2-ΔN169 /vMyx-ΔN169
VV-MT2-ΔE174 /vMyx-ΔE174
VV-MT2-ΔY236 /vMyx-ΔY236
VV-MT2-ΔI249 /vMyx-ΔI249
VV-MT2-ΔI260 /vMyx-ΔI260
VV-MT2-ΔI279 /vMyx-ΔI279
VV-MT2-ΔD303 /vMyx-ΔD303
VV-MT2ΔCRD1
VV-MT2ΔCRD2
VV-MT2ΔCRD3
VV-MT2ΔCRD4

Figure II-2. Construction of M-T2 C-terminal Deletions

Diagrammatic representation of the construction of M-T2 C-terminal unidirectional deletions, as described within the text, showing the M-T2 gene (rectangular box) and multiple cloning site within pMTN-6; H, Hind III; S, Sal I; P, Pst I; Xh, Xho I; Xb, Xba I; B, Bam HI.


P
Xh
Xb
S
B
M-T2 sequence
S
pMTN-6
S
PB
pMTN-6ΔXba/Xho
Xba Linkers
Xba Linkers
M-T2 Sequence
pMTN-6
Cys rich repeats
pMTN-ΔL113
pMTN-ΔN169
pMTN-ΔE174
pMTN-ΔY236
pMTN-ΔI249
pMTN-ΔI260
pMTN-ΔI279
pMTN-ΔD303

1. Digest with Pst I, Sal I
2. Treat with Exo III
   Nuclease S1
   Klenow
3. Add Xba I linkers,
   Digest with Xba I
4. Ligate

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CHAPTER III - SPECIES SPECIFICITY OF THE MYXOMA VIRUS TNF-RECEPTOR HOMOLOG[1]

INTRODUCTION

The TNF family, along with the IFNs, are critically important cytokines in establishing an early defense against viral infection (1, 2). In addition to mediating immune responses and inflammation, TNF can be directly cytotoxic to cells infected with certain viruses, such as vesicular stomatis virus, HSV and Newcastle disease virus (3-6). Many uninfected cells are particularily sensitive to direct lysis by TNF upon treatment with protein synthesis inhibitors (7). Shut-off of host protein and RNA synthesis often occurs in virally-infected cells, and the mechanism of cytolysis of infected cells by TNF may be similar to the *in vitro* TNF sensitivity observed in some cell types in the presence of inhibitors of macromolecule synthesis (1). TNF can thus exert antiviral effects by establishing and orchestrating the host immune response and also by directly inducing a cytolytic/apoptotic response in infected cells.

Myxoma virus infects the skin of rabbits and then migrates from the primary site of infection to establish a systemic infection which is poorly recognized by the host immune system and is invariably fatal for immunocompetent European rabbits (8-11). Inactivation of both copies of the myxoma T2 gene with a dominant selectable marker resulted in dramatic disease attentuation in infected rabbits such that the majority of rabbits infected with the T2-knockout virus were able to mount an effective immune response and recover from infection (12). In this communication we report on the role of TNF as an anti-poxviral cytokine and on the ability of myxoma T2 protein to counteract TNF activities. We have also investigated the species-specificity of TNF from several vertebrates. The anti-viral effects of rabbit TNF on poxviral replication in rabbit cells in particular was investigated, and the ability of myxoma T2 to inhibit rabbit, human and murine TNF expressed from isogenic vaccinia virus vectors was examined.

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RESULTS

Expression of Myxoma T2 Protein from a Recombinant Vaccinia Virus

In order to characterize the relationship between TNF and the myxoma T2 protein, over-expression of secreted M-T2 protein was required because in native supernatants from myxoma-infected cells M-T2 is only a minor component of the total secreted proteins (12). A recombinant vaccinia virus was therefore constructed in which the myxoma T2 gene, normally expressed as an early gene, was placed under the control of a strong, synthetic late promoter. Although vaccinia (strains Cogenhagen and WR) encodes two TNF receptor-like genes within its genome, both copies are present as discontinuous open reading frames which could only direct the synthesis of truncated proteins with incomplete TNF-binding domains (13, 14). Therefore, vaccinia WR was a suitable vector with which to express the myxoma T2 to evaluate putative TNF-inhibitory activities. The complete myxoma T2 coding sequence was subcloned into the vector pMJ602 (15) which targeted insertion of the myxoma T2 gene into the thymidine kinase *(tk)* locus of vaccinia virus WR. The structure of the recombinant virus thus generated, VV-MT2 is outlined in Figure III-1, and the putative 16 amino acid leader sequence, potential N-linked glycosylation sites and the cysteine rich repeats that are characteristic of members of the TNF-receptor superfamily are shown.

To determine whether the recombinant VV-MT2 virus directed the proper secretion of M-T2 protein, infected BGMK cells were labeled with [^{35}S]-methionine/cysteine for 1 hour at various times and the labeled polypeptides that were secreted during a one hour chase were collected and analyzed by SDS-PAGE. As shown in Figure III-2A, the profiles of proteins secreted at early times of infection was similar between cells infected with control vaccinia (lanes 1,2) and T2-expressing vaccinia virus (lanes 5,6). However, a major diffuse 55-60 kDa polypeptide was secreted into the medium by 6 to 8 hours post- infection from VV-MT2 infected cells that was absent in supernatants from control virus infected cells (compare lanes 3 and 7). Sequence analysis of the myxoma T2 protein predicted that cleavage between amino acid residues 16 and 17 would generate only a 33 kDa processed protein (12). However, four potential N-glycosylation sites are predicted from the myxoma T2 sequence and this discrepancy in size most likely resulted from glycosylation of the T2 protein. Similar glycosylation levels have been reported for another secreted myxoma protein, Serp1 (16). This 55-60 kDa T2 species represented the most adundantly labeled protein secreted from infected cells at late times (compare lanes 4 and 8 in Fig. III-2A) and significant amounts of M-T2 protein were also confirmed by the Coomassie staining profile of total secreted proteins (data not shown). The level of secretion of myxoma T2 protein from VV-MT2 infected cells was between one and two orders of magnitude higher than that observed with wild-type myxoma infected cells (data not shown), indicating that the recombinant vaccinia viruses still effectively utilized the processing/glycosylation pathway of the infected cell and that M-T2 secretion levels were not inherently limited by any sequence motifs of the M-T2 protein itself.

Expression of TNF from Different Species using Recombinant Vaccinia Viruses

In order to quantitate the relative activity profiles of three different vertebrate TNFs with each other, the rabbit, human and mouse ligands were expressed from identical vaccinia vehicles under the control of a common, synthetic late promoter (see Fig. III-1). The various TNF cDNAs were prepared as described in the Materials and Methods. The recombinant viruses, VV-RaTNF, VV-HuTNF and VV-MuTNF expressing rabbit, human and mouse TNF, respectively, are outlined in Figure III-1, which also illustrates the leader sequences, conserved cysteines and a single, potential N-linked glycosylation site in mouse TNF (17).

To determine whether BGMK cells infected with the recombinant vaccinia viruses directed the cleavage and secretion of the mature TNF ligands, supernatants prepared from infected cells were analyzed by Western blotting. As shown in Figure III-2B, a cross-reacting polyclonal rabbit anti-mouse TNF antibody recognized secreted TNF from all three species, although as expected, it had the greatest affinity for mouse TNF. The antibody did not recognize any proteins in supernatants from control VV-601 infected cells (Fig. III-2B, lane 4). In supernatants from cells infected with VV-RaTNF, the antibody recognized a 17.9 kDa polypeptide, which corresponds to the reported 18 kDa for mature rabbit TNF monomer, when analyzed by denaturing SDS-PAGE (17). The mobility of rabbit TNF was identical to the mobility of purified recombinant human TNF prepared in *E. coli*. The major 16 kDa band recognized by the anti-TNF antibody in supernatants from infected cells expressing mouse or human TNF had a slightly faster mobility than the purified recombinant human TNF. Mouse TNF monomer has been reported to migrate as a 16-18 kDa band on SDS polyacrylamide gels, whereas human TNF monomer migrates as a 17 kDa band (17). This discrepancy in size may reflect minor alterations in processing of the precursor peptide. TNF is first synthesized as a 26-kDa integral transmembrane protein with a 75 amino acid, N-terminal precursor sequence which anchors the protein to the plasma membrane (reviewed in Fiers, 1992). Proteolytic cleavage by an unidentified protease then releases the mature 17 kDa polypeptide from the membrane as bioactive trimers. The vaccinia viruses expressing rabbit, human and mouse TNF encode the entire open reading frame and secretion of TNF from virally-infected cells should thus mimic the *in vivo* secretion pathway. In supernatants from cells infected with VV-HuTNF, two 16-18 kDa species of human TNF were observed which most likely corresponds to two processing events near the predicted cleavage site. A minor 21 kDa mouse TNF peptide was also observed although at very low levels but it is not known if this is a result of inefficient cleavage or other post-translational modifications. It is still uncertain whether these apparently alternative forms of TNF are equally biologically active as mature trimers although the first nine amino acids of the mature human TNF can be deleted without affecting TNF activity when assayed by cytolysis of sensitive cells (18).

The secreted TNF present in concentrated supernatants prepared from virally-infected roller bottle cultures was quantitated by scanning the TNF bands visualized by silver staining of polyacrylamide gels, using standardized recombinant human TNF as a control. The mass of TNF secreted from recombinant virus infected cells was uniformly lower than the amount of M-T2 protein present in supernatants prepared from comparable VV-MT2 infected cells. At 24 hours post-infection, M-T2 was present in the native supernatant at 26 µg/ml (5.0×10^{-10} mol/l of monomer peptide, 18% of total supernatant protein by mass), compared to 2.2 µg/ml human TNF (1.25×10^{-10} mol/l, 10% of total mass), 1.9 µg/ml mouse TNF (1.1×10^{-10} mol/l, 5.9% of total mass), and 50 pg/ml rabbit TNF (3.0×10^{-12}mol/l, 0.12% of mass). The supernatants used for these experiments were prepared from infected BGMK cells, but the overall secretion levels of the different TNFs might well vary according to the type of infected cell.

Growth of Recombinant TNF-Expressing Viruses in Tissue Culture

Since TNF is known to have direct antiviral effects, the consequences of expressing TNF from a vaccinia viral vector on the growth of that virus in tissue culture was tested in various cell lines. Monkey BGMK cells, rabbit RK-13 cells, rabbit SIRC cells and mouse L929-8 cells were infected with the different viruses at low m.o.i., and the progeny yield after 48 hours was determined. As shown in Figure III-3, expression of any of the TNFs did not appreciably affect the virus yield in BGMK, RK-13 or SIRC cells. At the different multiplicities tested, the yield of VV-TNF expressing viruses was comparable within experimental error to the virus yield from cells infected with vector control virus VV-601. Interestingly, replication of the recombinant TNF-expressing viruses in mouse L929-8 cells was significantly reduced when compared to vector control virus, VV-601 (Fig. III-3D). Presumably, the endogenous TNF produced by the recombinant viruses caused premature cell lysis before the viral replicative cycle was complete. This attentuation was the greatest for VV-MuTNF, which expresses the homologous mouse TNF, and least for the vaccinia virus expressing rabbit TNF.

Biologically Active TNF is Secreted from VV-TNF Infected Cells

To determine whether biologically active TNF was produced from cells infected with the recombinant vaccinia viruses, virus-free supernatants from infected cells were assayed for TNF cytolytic activity using the TNF-hypersensitive cell line L929-8 as indicators of TNF bioactivity. In the presence of actinomycin D, mouse L929-8 cells are efficiently lysed within 18 hours of treatment with TNF, with a limit of detection of 200 fg/ml for mouse TNF (19). These cells have been reported to be insensitive to all other cytokines tested (19).

Concentrated virus-free supernatants prepared from cells infected with control vaccinia virus were first tested to ensure that vaccinia infected cells did not secrete any TNF-like activities. Actinomycin-sensitized mouse L929-8 cells, monkey BGMK cells, rabbit RK-13 and SIRC cells were incubated with various dilutions of concentrated viral supernatant, and their viability assayed 18 hours later. As shown in Figure III-4A, cells from all four cell lines remained fully viable at all concentrations tested.

Supernatants containing rabbit, mouse or human TNF were assayed for biological activity by cytolysis of L929-8 cells. As shown in Figure III-4B, L929-8 cells were extremely sensitive to supernatants prepared from each of the VV-TNF infected cells, indicating that biologically active TNF was secreted in each case. The cells were most sensitive to cytolysis by the homologous mouse TNF, and least sensitive to rabbit TNF. The LD_{50} for rabbit TNF was 16 pg/ml, indicating that these cells were about 20-fold less sensitive to cytolysis by the heterologous rabbit TNF than mouse TNF. The LD_{50} of 4 pg/ml for human TNF and 0.8 pg/ml for mouse TNF from viral supernatants compares favourably with the previously characterized sensitivity of these cells for purified recombinant human TNF (3.6 pg/ml) and purified recombinant mouse TNF (0.6 pg/ml) (19).

Sensitivity of Cell Lines to Cytolysis by TNF

Cell lines vary considerably in their sensitivity to TNF. When active, TNF can be either cytostatic (growth inhibitory) or cytolytic upon sensitization with protein synthesis inhibitors (7). To determine which cell lines were sensitive to TNF and to test the species-specificity of rabbit, mouse and human TNFs, the cytolytic activity of the different TNFs were assayed on monkey epithelial BGMK cells, rabbit epithelial RK-13 cells, and rabbit corneal, fibroblastoid SIRC cells. Cells were treated with actinomycin D and various TNF concentrations, and the percentage of viable cells remaining after 18-20 hours was measured.

As seen in Figure III-5A, monkey BGMK cells were relatively insensitive to human, mouse or rabbit TNF at concentrations of up to 0.1 μg/ml. Although the homologous primate TNF was not used, it would be expected that TNF-sensitive primate cells would be comparably sensitive to cytolysis by human TNF.

On the other hand, the rabbit cell lines RK-13 and SIRC were susceptible to direct cytolysis by rabbit TNF. The data from two independent titration experiments showing the killing profile of RK-13 cells in response to rabbit TNF is shown in Figure III-5B. The LD_{50} of these cells to rabbit TNF was 2 ng/ml (120 pmol/l). The TNF-sensitive L929-8 cell line was 125-fold more sensitive to lysis by rabbit TNF than RK-13 cells and demonstrated a steeper dose-response curve to TNF. RK-13 cells were completely insensitive to cytolysis by human or mouse TNF, even at concentrations of 1 μg/ml (Fig. III-5B, upper right). This result is rather surprising, as the cytolytic activity of TNF has been characterized as being relatively species non-specific (17).

Rabbit TNF also displayed a cytotoxic effect on rabbit SIRC cells (Fig. III-5C) with an LD_{50} of 2 ng/ml. Moreover, like rabbit RK-13 cells, SIRC cells were also completely insensitive to the effect of human and mouse TNF at concentrations even up to 1 μg/ml, suggesting that rabbit cells demonstrate species-specificity in their response to TNF and are only sensitive to the cyolytic effects of the homologous rabbit cytokine.

Rabbit TNF is Cytostatic to Rabbit RK-13 Cells

In the absence of inhibitors of macromolecule synthesis, TNF can also kill certain hypersensitive cell lines, but the mechanism of this cytotoxicity is thought to be different than that of the rapid cytolytic response described above, as cell death occurs on a slower time scale, over the course of several days (7). To determine whether the rabbit cell lines were sensitive to TNF even in the absence of actinomycin D, RK-13 and SIRC cells seeded at low density were incubated with various concentrations of rabbit TNF and after 1-5 days, the number of dividing viable cells quantitated by uptake of the vital dye neutral red. Typically, after 3 to 4 days, untreated and TNF-insensitive cells reach saturation density, while TNF-sensitive cells show a concentration-dependent growth inhibition and eventually die (Fig. III-6). The response of RK-13 cells to rabbit TNF in the absence of actinomycin D (Panel A) was typical of cells sensitive to the cytostatic/cytotoxic properties of TNF. After 1-2 days of exposure to TNF, a 10-20% decrease in the number of viable cells was observed at the highest TNF concentrations, which becomes much more pronounced by day 3, when the number of viable cells was less than 50% that of untreated cells. The cell monolayer appeared intact after 3 days (data not shown), but by days 4 to 5, the cells had started to disintegrate indicating cell death had begun. By this time, the number of viable cells had dropped to less than 10% of the untreated control. Supernatants from cells infected with vector control virus, however, had no effect on RK-13 cells even after 5 days (Fig. III-6A), indicating the cytotoxic activity within the supernatants was due to the expressed rabbit TNF. At day 5, a 50% decrease in the number of viable cells was observed at a TNF concentration of 250 pg/ml.

Although SIRC cells were sensitive to cytolysis by rabbit TNF, they were relatively insensitive to TNF in the absence of actinomycin D (Fig. III-6B). After 2 days treatment with 10 ng/ml rabbit TNF, no effect was seen on the number of viable cells. At 4 to 5 days of TNF treatment, the amount of viable cells was still 60% of untreated control cells, which suggests that TNF might have a slight cytostatic effect on these cells but clearly, however, they are not nearly as sensitive as RK-13 cells to the cytostatic/cytotoxic effects of TNF.

Rabbit TNF has Only a Modest Anti-Viral Effect on Poxvirus-Infected RK-13 Cells

Since the RK-13 cell line was found to be extremely sensitive to cytolysis by rabbit TNF in both the presence and absence of protein synthesis inhibitors, this represented a useful TNF-responsive cell line to evaluate the antiviral capacity of TNF on poxviruses in cultured rabbit cells. To mimic the cell-cell spread of myxoma virus at sites of infection, semi-confluent monolayers of RK-13 cells in six-well dishes were infected with a total of 50 p.f.u. vaccinia or myxoma virus. Rabbit TNF (0-1 ng/ml) was added, and 72 hours post infection, cells were harvested and virus titre determined by plaque assay. The mean virus yield for both vaccinia (VV-601) and the recombinant virus expressing myxoma T2 (VV-MT2) was reduced by 50% in the presence of 1 ng/ml TNF (Fig. III-7, Panel A). In this experiment, a vast excess of TNF would be present compared to the mass of T2 protein actually secreted from the initial fifty infected cells. Similar results were observed with RK-13 cells infected with myxoma virus or the T2-knockout myxoma virus (Fig. III-7, Panel B). These results demonstrate that rabbit TNF does have a modest effect on the growth of both myxoma and vaccinia in RK-13 cells but because of the vast excess of TNF compared to T2 protein produced, any inhibition of the rabbit TNF by T2 protein could not be evaluated in this protocol.

Myxoma T2 Protein May Provide Modest Protection Against Anti-Viral Effects of TNF

Although rabbit TNF had a modest antiviral effect on poxviral replication in RK-13 cells in the previous experiment, myxoma T2 protein did not appear to protect against

TNF, presumably because TNF was in vast excess. To see if M-T2 protein could inhibit an antiviral effect of TNF in TNF-hypersensitive cells, L929-8 cells were co-infected with VV-MT2 and VV-RaTNF at ratios of 9:1 at a m.o.i. of 10, or 1:1 at a m.o.i. of 0.2, and virus titers determined after 24 hours and 48 hours respectively. Here, the vaccinia viruses were driving the expression of both rabbit TNF, which would induce an antiviral effect on sensitive cells, and myxoma T2 protein, which presumably would bind and sequester the secreted TNF. This experiment attempts to mimic the *in vivo* situation in which infected cells secrete M-T2 and immune cells produce TNF. Two ratios of VV-MT2 to VV-RaTNF were tested, to see if an excess of T2 was required to observe an inhibitory effect. The results are shown in Figure III-8. As expected, VV-RaTNF did not replicate as efficiently as VV-601 (Fig. III-8B, and compare with Fig. III-3D). A modest inhibition of the antiviral effect of expressing rabbit TNF from a vaccinia virus vector was observed, however, when cells were co-infected with VV-MT2 that was not observed when cells were co-infected with control VV-601 at both an m.o.i. of 10 and 0.2. However, there was considerable variation in the virus yields between samples, and the differences observed could also be attributed to the upper limits of experimental error. To confirm that this result is valid, the experiment would have to repeated several times, with larger sample sizes.

The Myxoma M11L and SERP-1 Gene Products Do Not Inhibit the Anti-Viral Effects of Rabbit TNF

Since TNF only appears to have a modest antiviral effect on the replication of myxoma virus and vaccinia, these viruses may be encoding other factors which interact with effectors of a TNF response downstream of ligand:receptor interaction. For example, the coxpox *crmA* gene product, a serine proteinase inhibitor which inhibits interleukin-1β converting enzyme (ICE), is also able to inhibit Fas- and TNF-induced apoptosis (20, 21). SERP-1, is a secreted myxoma protein with homology to serine proteinase inhibitors, and may be able to interact with a proteinase such as ICE or an ICE-like proteinase important in mediating a cytolytic response (22, 23). Although the cellular target for M11L has not been identified, it is an important virulence factor for the virus, and has an anti-apoptotic effect in rabbit lymphocytes (16, 24). To see if expression of the *M11L* or *SERP-1* genes was providing protection against the antiviral effects of TNF, RK-13 cells were infected with vMyxlac or myxoma viruses inactivated in the M-T2, M11L or SERP-1 genes at a m.o.i. of 0.1, in the presence or absence of 1 nM rabbit TNF, and virus titers determined after 48 hours. TNF had an antiviral effect on the replication of all the myxoma constructs in RK-13 cells, and virus yields decreased 2.5 to 4-fold in the presence of TNF (Fig. III-9) However, there was little difference in the virus yields of vMyxlac and myxoma viruses inactivated in the *M-T2*, *M11L* and *SERP-1* genes, indicating that these gene products, by themselves, are not protecting against the antiviral effects of TNF.

Myxoma T2 Protein Inhibits Rabbit TNF but not Human or Mouse TNF

To test the model that the myxoma T2 protein acts *in vivo* to bind and thus inactivate rabbit TNF, the effect of M-T2 protein on inhibiting cytolysis of L929-8 cells by the various TNFs was tested. Although RK-13 cells were also sensitive to rabbit TNF, L929-8 cells were chosen for these assays because they displayed a steeper dose-response curve to TNF and were sensitive to the action of each of mouse, human and rabbit TNF. For the rabbit TNF-protection assay, actinomycin D-sensitized L929-8 cells were co-incubated with rabbit TNF at concentrations which gave a percent killing of >95% (5-250 pmol/l) and cell viability was monitored in the presence of increasing molar excess of T2 protein. The percent viability of cells at the different T2:rabbit TNF ratios is shown in Figure III-10. Note that the molar ratios of T2 to TNF were calculated based on monomer size of each protein. No protection against cytolysis was observed at T2:TNF ratios of <1, when excess rabbit TNF over T2 protein was present. However, as the amount of T2 protein was increased, L929-8 cells were progressively protected from cytolysis by rabbit TNF until 100% protection was observed under ratios of T2:TNF >1000.

The ability of the myxoma T2 protein to inhibit cytolysis by mouse and human TNF was also tested and the results are summarized in Table III-1. Two concentrations (4 and 40 pg/ml) of these TNFs were chosen to provide a window to identify any protection by M-T2 against cytolysis. Since L929-8 cells show a sharp dose-response to TNF, the ability of T2 protein to inactivate even modest amounts of mouse TNF should lower the effective TNF concentration and would result in increased cell viabiltiy. However, absolutely no increase in L929-8 cell viability was observed even at T2:TNF ratios as high as 40,000:1. Similar results were observed with human TNF. At 4 pg/ml, L929-8 cells exhibited 80.2 % viability, and at T2:TNF ratios of 40,000, the viability observed (71.9%) was still within experimental error. In these experiments T2 protein and TNF were added simultaneously. Two-hour pre-incubation of M-T2 and TNF before addition to L929-8 cells had no effect on the ability of M-T2 to protect against cytolysis by TNF (data not shown). Thus, it appears that the M-T2 protein demonstrates strict species specificity and is capable of inhibiting the biological activity only of rabbit TNF.

DISCUSSION

Poxviruses use various mechanisms to inactivate cytokines and evade host immune responses (25). The importance of ligands of the TNF family in counteracting poxviral infection is attested to by the attentuation of a recombinant myxoma virus in which both copies of the TNF receptor analogue gene, designated M-T2, had been inactivated (12). Myxomatosis, the virulent disease syndrome caused by myxoma virus in European rabbits, is a useful model to investigate virus-host interactions (8). The disease has been well characterized in the rabbit, and effects of myxoma virus gene alterations on virus virulence are readily studied *in vivo* (8-10, 26). Three types of TNF ligand are now recognized in the growing TNF superfamily: TNF, LTα (TNFβ), and LTβ (27). In this communication we have investigated the interaction between TNF and the myxoma T2 protein, and the role of TNF in inhibiting poxvirus replication. The secreted TNFs produced from isogenic vaccinia virus vectors were used to study the species-specificity of mouse, human and rabbit TNF. In general, the biological activities of TNF have not been characterized as species-specific, although some species preference has been reported. Human, mouse, rabbit, ox and horse TNF have all been found to display cytolytic activity against TNF-sensitive mouse L929 or WEHI164 clone 13 cells (19, 28-30). TNFs are highly conserved amongst human, pig, cow, rabbit and rat, with sequence homologies greater than 75% (31), and this is reflected in the ability of a variety of heterologous TNFs to mediate a cytolytic signal through the mouse TNF receptors. Using TNF expressed from vaccinia viral vectors, we observed some species preference in that mouse L929-8 cells showed a 5-fold decreased sensitivity to human TNF over mouse TNF and a 20-fold decreased sensitivity to rabbit TNF. This lack of species restriction by L929-8 cells allowed this cell line to be used to evaluate and contrast the ability of myxoma T2 protein to inactivate a variety of different vertebrate TNFs.

Related but divergent cellular receptors for TNF, denoted as either Type I and II or as p55 and p75, have been identified and cDNAs cloned from mouse and human cells (32-34). The mouse and human p55 receptors bind both mouse and human TNF, whereas the mouse p75 receptor binds only mouse TNF (32). Many of the biological activities of TNF important for cytotoxicity, antiviral effects, induction of superoxide dismutase and NF-κB, and cytolysis of TNF-sensitive cells are thought to be mediated via the p55 TNF receptor (35-37). The p55 receptor signaling by TNF is thought to be species non-specific, which accounts for the biological activity of TNFs from different species on mouse and human cells (17, 32). More stringent species specificity has been observed for activities attributed to the signaling of the p75 receptor. For example, proliferation of mouse thymoctyes and the stimulation of the murine cytotoxic T-cell line

CT-6 is specific for mouse TNF and is thought to be mediated by the p75 receptor (38). Very recently, a third cellular receptor with specificity for cell associated lymphotoxin-β has also been discovered but its role(s) in TNF responsiveness remains to be investigated (39).

In this communication we report that the permissiveness of mouse TNF receptors for TNFs of different species is distinctly different from that of the rabbit cellular TNF receptors. Rabbit RK-13 and SIRC cells were only sensitive to cytolysis by rabbit TNF, and if TNF signaling in rabbit cells follows similar pathways to that found in human and mouse, we predict that the rabbit p55 TNF receptor will engender this specificity for rabbit TNF. Species variations in TNF sensitivity undoubtedly reflect divergence in protein sequence and structure for both the cytokine and the receptor. TNF is highly conserved amonst the known vertebrate species examined to date . For example, mouse and human TNF are 80% identical whereas mouse and rabbit TNF share 78% identity (31). Despite these sequence similarities, rabbit, human and mouse TNFs are all active on murine cells, while mouse and human TNF are completely inactive on rabbit cells. The rabbit TNF receptors have not been cloned and so the extent of sequence divergence at the receptor level is unknown.

Poxviruses encode proteins with homologies to a number of different cytokine receptors (13, 40, 41) . Presumably, these proteins bind and inhibit cytokine activities, downregulating the host immune response to the virus infection. These viral cytokine binding proteins in many ways ressemble the soluble shed forms of the native cellular receptors which are speculated to modulate cytokine activity within the host organism (42, 43). Soluble TNF receptors that possess intact extracellular ligand binding domains have been detected in blood, urine and other biological fluids and act as natural inhibitors of TNF signaling (44-47). By competing with cell surface receptors, they are thought to lower the effective concentration of soluble TNF and protect the organism against generalized toxicity associated with high TNF levels. Purified secreted TNF receptors have been shown to inhibit the cytotoxic activity of TNF on mouse cells *in vitro* (44) and *in vivo* can protect mice against endotoxemia, inhibit acute inflammation in rats and impede the development of arthritis in a mouse arthritis model (48-50). The myxoma T2 viroceptor most likely acts in an equivalent fashion to dampen all TNF activities that depend on TNF-receptor triggering, both antiviral and immunomodulatory. Here we show that the T2 protein of myxoma virus inhibits the cytolytic activity of rabbit TNF on TNF-sensitive target cells. Presumably, T2 binding to TNF directly prevents its interaction with host receptors, but the possibility that M-T2/TNF complexes could still bind receptors but fail to signal has not been formally excluded.

The T2 protein of the closely related Leporipoxvirus, SFV, expressed from COS cells, has previously been shown by ligand blotting to bind human TNF/LTα (51), but our data indicate that the T2 protein of myxoma cannot inhibit the biological activity of either the human or mouse ligands. Scatchard analysis of the binding between these T2 proteins and the TNFs of rabbit, mouse and human will be useful in order to resolve this apparent discrepancy.

Recombinant vaccinia viruses that direct the synthesis of rabbit, mouse or human TNF were significantly attenuated in the TNF-hypersensitive line L929-8. Presumably, TNF produced by the virus vector is prematurely lysing infected cells before the virus replication cycle is complete. Surprisingly, rabbit TNF had only a modest inhibitory effect on the growth of both vaccinia and myxoma viruses in cultured rabbit cells, whether or not the T2 gene was expressed (see Fig. III-7). TNF can directly kill some virally-infected cells and is capable of inducing an antiviral state in uninfected cells (2, 52) but it should be emphasized that TNF is also an important cytokine in establishing an immune response and mediating the inflammatory response to a challenge by many pathogens (53, 54). Thus, there are multiple reasons to explain why a virus might target TNF for virus-specific countermeasures. The two- to four-fold reduction in vaccinia and myxoma yields on RK-13 in the presence of cytotoxic levels of TNF demonstrates that

TNF can have a direct effect on limiting virus growth in tissue culture, but on the other hand, by shutting off host protein synthesis, one might predict that poxvirus-infected cells would become hypersensitive to lysis by TNF, especially in the case where the only known anti-TNF gene, M-T2 has been deleted in vMyxt2g. However, it cannot be ruled out that poxviruses use additional strategies to interfere with TNF-signaling downstream of ligand/receptor interactions. The vaccinia virus strain WR, used in these experiments, has recently been shown to encode a homolog, *B13R*, of the cowpox inhibitor of Fas- and TNF-mediated apoptosis, crmA (13, 14, 21). Whether the B13R gene product actually inhibits TNF-cytolytic pathways is unknown, but it is conceivable that the B13R protein was acting to inhibit TNF-mediated lysis of infected cells, thus allowing for productive viral infection. These results suggest that poxviruses might very well encode other proteins which directly interfere with the TNF-induced apoptotic signal in a fashion reminiscient of adenovirus proteins which interfere with TNF-induced cytolysis (55, 56). The myxoma *SERP-1* and *M11L* gene products do not appear to inhibit rabbit TNF signaling events in that myxoma constructs in which either of these genes were inactivated were not hypersensitive to the antiviral effects of TNF.

The T2 protein of myxoma contributes to viral virulence in infected rabbits and inactivation of the T2 gene within myxoma virus resulted in significant attenuation of the virus (12). TNF receptor homologues are also found in some orthopoxviruses, in particular cowpox and variola, but vaccinia virus (strains WR and Copenhagen) contains two fragmented TNF-receptor homologues that are each present as discontinuous open reading frames that could only express truncated proteins with incomplete TNF-binding domains (13, 14). Thus, in the process of extensive passage outside its native host, one of the virulence factors vaccinia has lost is its TNF receptor cognate. Recently, it has been discovered that one particular strain of vaccinia (Lister) may have retained a functional TNF receptor homologue (A. Alcami and G. Smith, personal communication).

It is still formally unproven whether viral cytokine receptor genes have originated from the genome of an infected host or have independently evolved by convergent evolutionary pressure to recognize a given cytokine. If a given poxvirus has acquired a host TNF receptor gene and then modified it to express only the truncated, soluble form, it is predicted that the modified virus would have a selective growth advantage in a vertebrate host capable of a TNF response. We still do not know whether an ancestral poxvirus acquired a progenitor TNF-binding protein which subsequently evolved within modern day poxviruses to preferentially mirror the TNF-specificity of the natural host, or whether different poxviruses independently acquired the genes for the same cytokine receptor family. We have shown here that rabbit TNF receptors are specifically triggered by rabbit TNF, and myxoma T2 protein is only capable of protecting sensitized target cells from cytolysis by the rabbit TNF ligand. We suggest that the apparent inability of myxoma T2 protein to inhibit human and mouse TNF is provocatively similar to the low activity profile of human and mouse TNF ligands on rabbit cells, and predict that myxoma virus originally acquired a rabbit TNF receptor during its long symbiotic relationship (probably since the early Pleistocene) with the South American rabbit. Thus, we expect that careful analysis of the species specificity profiles of poxviral viroceptors such as M-T2 will shed light not only on the role of individual cytokines on viral pathogenesis, but also on the evolutionary history of individual poxviruses in terms of their vertebrate host origins.

Figure III-1. Structure of Recombinant Vaccinia Viruses

Myxoma T2, rabbit TNF, human TNF and mouse TNF coding sequences were placed under the control of a vaccinia synthetic late promoter (SLP), and inserted into the *th*ymidine kinase, *tk*, locus of vaccinia (WR). The vector control virus, VV-601 contains only lacZ inserted into *tk*. N-terminal leader sequences (⧅) with putative cleavage sites (↑), cysteine-rich repeats (□), cysteine residues (|), and potential N-linked glycosylation sites (*) are shown.

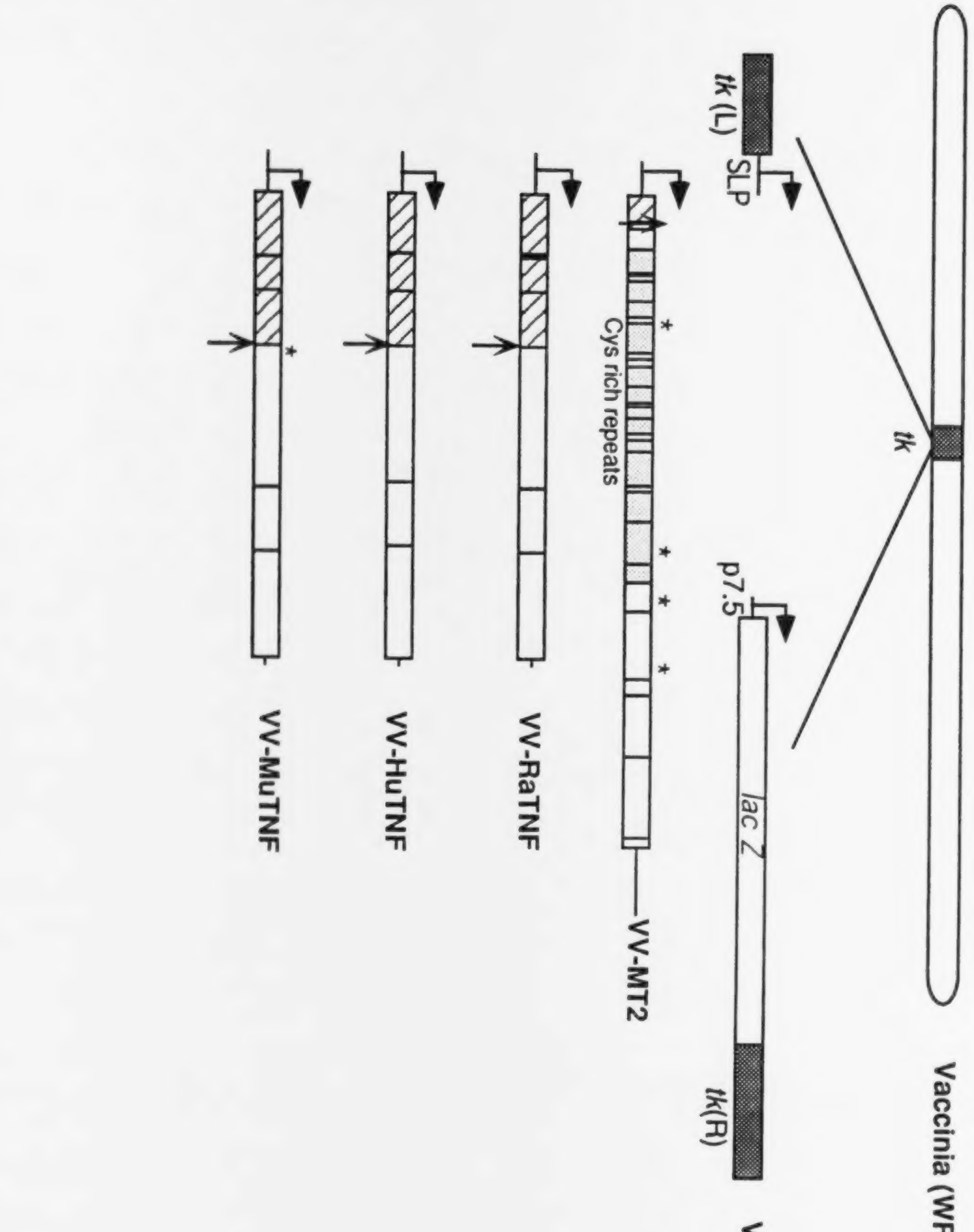
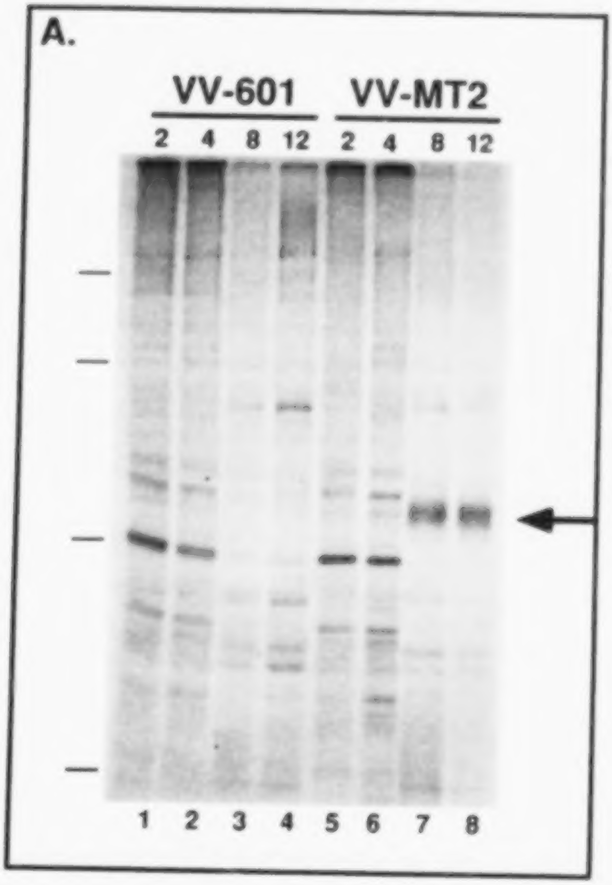

Vaccinia (WR)
tk
tk (L)
SLP
p7.5
lac Z
tk(R)
VV-601
Cys rich repeats
VV-MT2
VV-RaTNF
VV-HuTNF
VV-MuTNF

Figure III-2. M-T2 and Rabbit, Mouse and Human TNFs are Produced and Secreted from Recombinant Vaccinia Virus Vectors

A. Secretion of myxoma T2 from vaccinia-infected cells at late times. Cells were labelled with ^{35}S-Met/Cys as described under Materials and Methods and chased for 1 hour at early and at late times of infection. Secreted proteins were precipitated and analyzed by 10% SDS-PAGE. Lanes 1-4, VV-601, lanes 5-8, VV-MT2; harvest times were 2 (lanes 1,5), 4 (lanes 2,6), 8 (lanes 3,7) and 12 (lanes 4, 8) hours post infection. The arrow indicates, in lanes 7 and 8, the T2 protein secreted at late times from VV-MT2 infected cells, that is absent from VV-601 infected cells. The bars indicate the positions of marker proteins, 97.4, 66.2, 45.0, and 31.0 kDa, from top to bottom.

B. Secreted rabbit, mouse and human TNF in supernatants prepared from recombinant vaccinia virus infected cells. Supernatants were prepared as described, analyzed by SDS-PAGE, as above, and immunoblotted using anti-mouse TNF antibody. Supernatant samples were diluted 1/100 for mouse TNF and 1/20 for human TNF. Supernatants were from (1) VV-MuTNF (2) VV-HuTNF (3) VV-RaTNF (4) VV-601 control -infected cells and (5) purified recombinant human TNF α (50 ng), used as a standard. The bars indicate the positions of marker proteins, 66.2, 45, 31.0, and 21.5 kD, from top to bottom.


A.
VV-601
VV-MT2
2 4 8 12 2 4 8 12
1 2 3 4 5 6 7 8

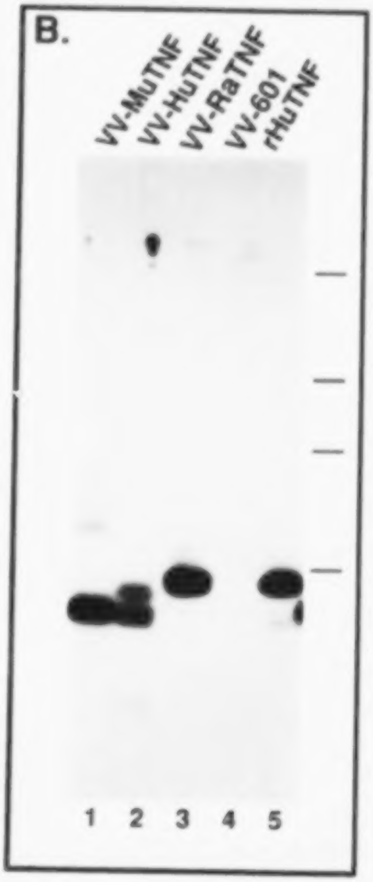

B.
VV-MuTNF
VV-HuTNF
VV-RaTNF
VV-601
rHuTNF
1 2 3 4 5

Figure III-3. Virus Yield from Cells Infected with Recombinant Vaccinia Viruses Expressing TNF

A.) BGMK, B.) RK-13, C.) SIRC and D.) L929-8 cells were infected with recombinant vaccinia virus at a m.o.i. of 0.1, 0.01, or 0.001 and the virus allowed to propagate for 48 hours. The cells were harvested and the virus titer determined by plaque assay on BGMK cells; VV-601 ( ), VV-RaTNF (░), VV-MuTNF (▧). VV-HuTNF (▩). The virus yield is expressed as the log of the ratio of p.f.u. after 48 hour culture/input p.f.u., and error bars designate average deviations of duplicate measurements.

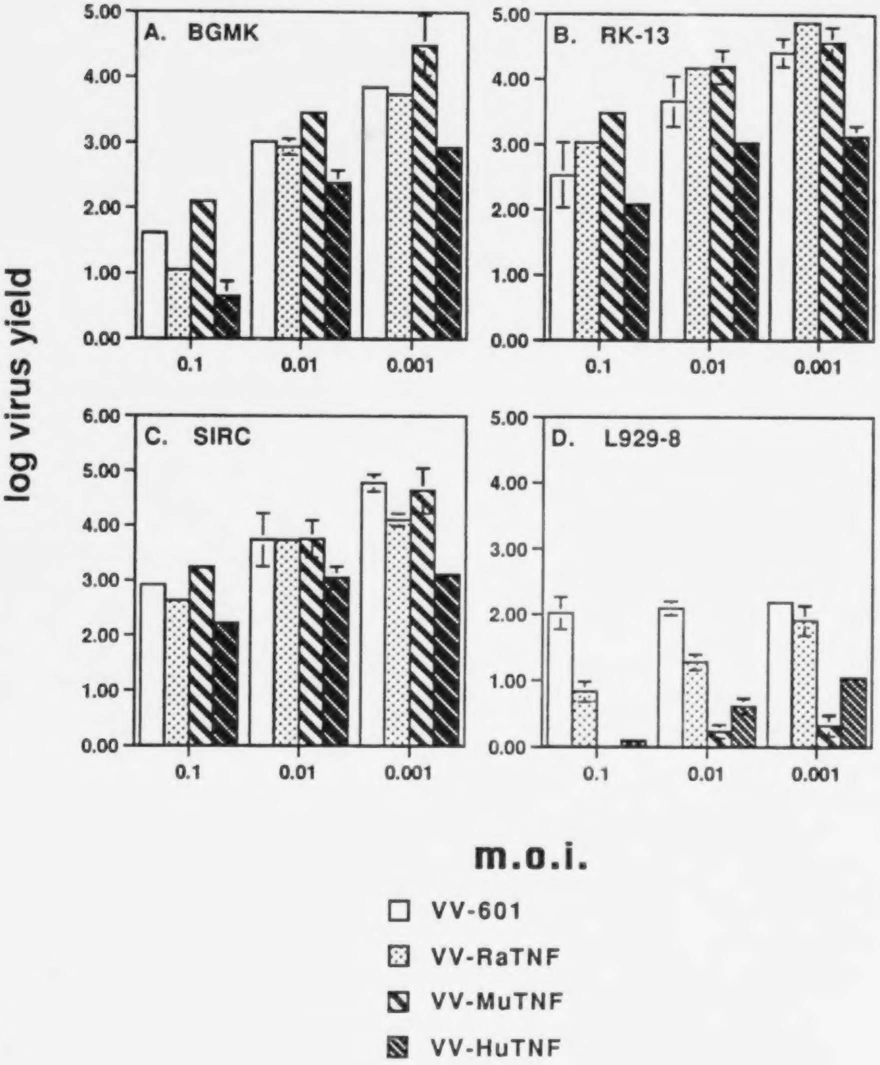

A. BGMK
B. RK-13
C. SIRC
D. L929-8
log virus yield
5.00
4.00
3.00
2.00
1.00
0.00
6.00
0.1
0.01
0.001
m.o.i.
VV-601
VV-RaTNF
VV-MuTNF
VV-HuTNF

Figure III-4. Biologically Active TNF is Secreted from Cells Infected with Recombinant Vaccinia Viruses

A. Cell viability in the presence of concentrated supernatants from vaccinia infected cells. Actinomycin-D sensitized L929-8 (□), BGMK (◇), RK-13 (○) or SIRC (△) cells were incubated with various dilutions of concentrated supernatants from VV-601 infected cells, prepared as described under Materials and Methods.

B. Biologically active recombinant TNF (rTNF) is secreted from cells infected with recombinant vaccinia viruses. L929-8 cells were treated with rabbit TNF (□), mouse TNF (○) or human TNF (△)-containing supernatants in the presence of 2 μg/ml actinomycin D. Cell viability was determined by neutral red uptake and results are presented as the % viability (see Materials and Methods). Each point represents the mean of quadruplicate determinations and standard deviations were <15% of the mean.

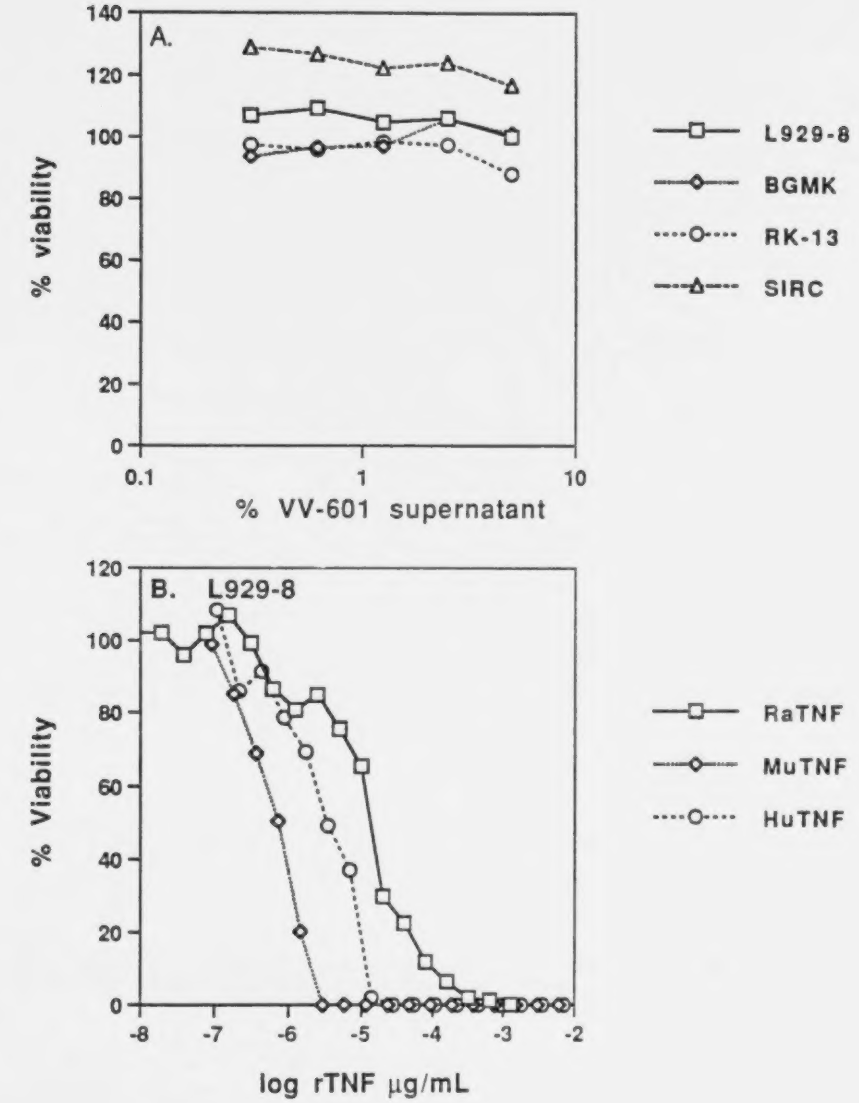

A.
% viability
140
120
100
80
60
40
20
0
0.1
1
10
% VV-601 supernatant
L929-8
BGMK
RK-13
SIRC
B. L929-8
% Viability
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
-2
log rTNF μg/mL
RaTNF
MuTNF
HuTNF

Figure III-5. Sensitivity of Cell Lines to the Cytolytic Effects of TNF

Cells were treated with recombinant TNFs (rTNF) in the presence of 2 μg/mL actinomycin D, and cell viability was determined by neutral red uptake. Results are presented as the % viability (see Materials and Methods). The effect of rabbit TNF (□), mouse TNF (○), and human TNF (▲) on A.) BGMK B.) RK-13 and C.) SIRC cells is shown. Results from duplicate experiments are shown for RK-13. Each point represents the mean of quadruplicate determinations and standard deviations were <15% of the mean.

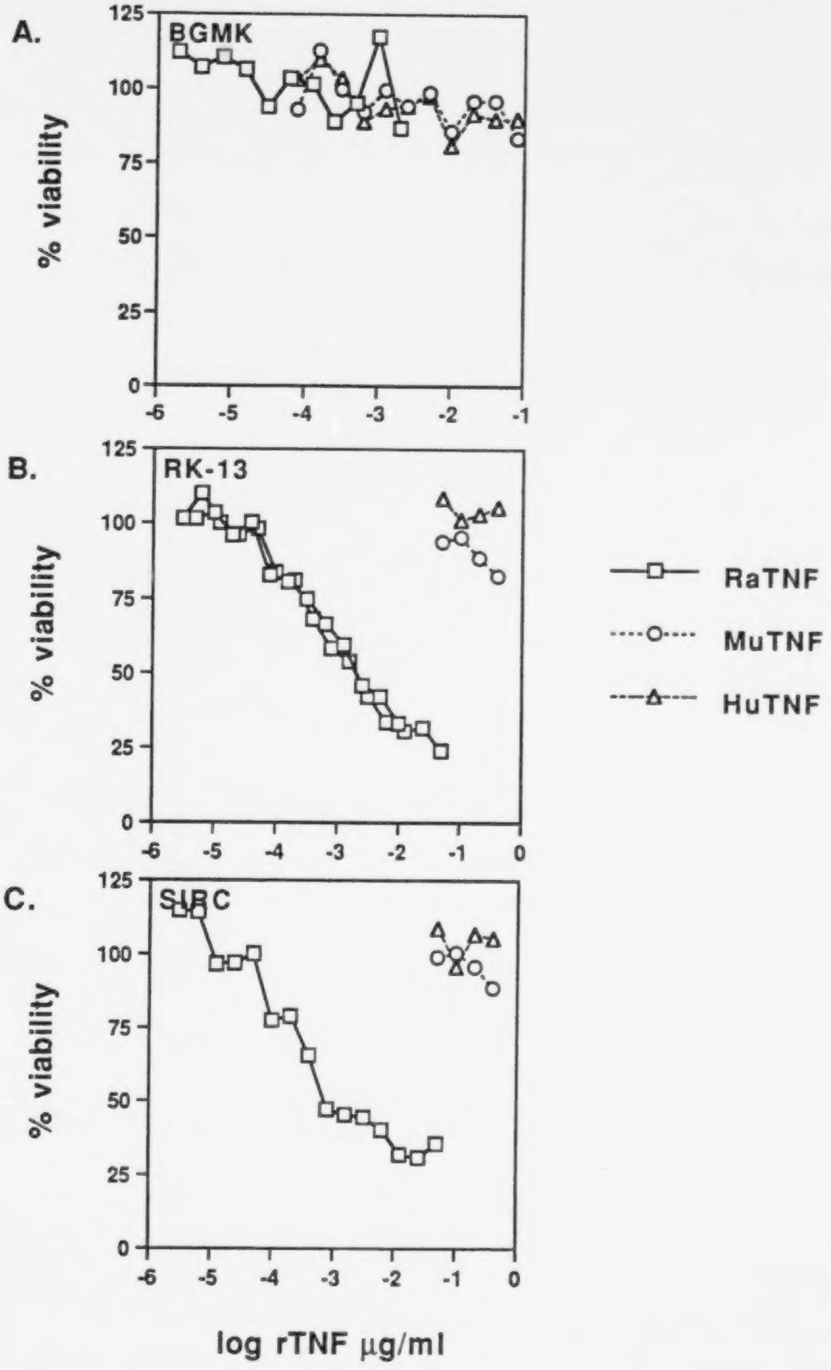

A.
BGMK
B.
RK-13
C.
SIRC
% viability
125
100
75
50
25
0
-6
-5
-4
-3
-2
-1
0
RaTNF
MuTNF
HuTNF
log rTNF µg/ml

Figure III-6. Cytostatic Effect of Rabbit TNF (RaTNF) on Rabbit Cells Lines

A. RK-13 or B. SIRC cells were incubated with various dilutions of rabbit TNF, or control supernatant for 5 days (◆), and viable cells assayed by neutral red uptake after 1 (□), 2 (◇), 3 (○), 4 (Δ) and 5 (⊞) days. Results are expressed as the % control viable cells, see Materials and Methods.

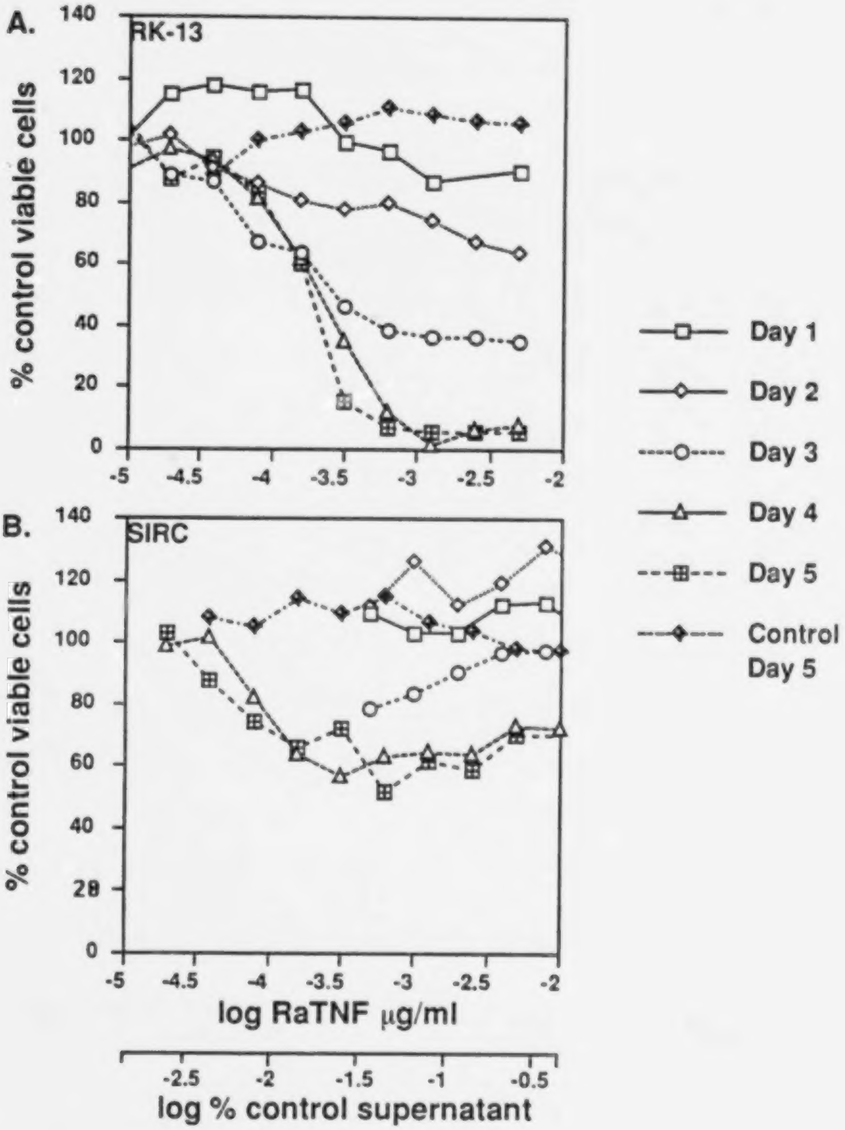

A.
RK-13
% control viable cells
140
120
100
80
60
40
20
0
-5
-4.5
-4
-3.5
-3
-2.5
-2
B.
SIRC
% control viable cells
140
120
100
80
60
40
20
0
-5
-4.5
-4
-3.5
-3
-2.5
-2
log RaTNF μg/ml
-2.5
-2
-1.5
-1
-0.5
log % control supernatant
Day 1
Day 2
Day 3
Day 4
Day 5
Control Day 5

Figure III-7. Effect of Rabbit TNF on Virus Yield of Vaccinia and Myxoma Viruses in Rabbit TNF-Sensitive Cell Lines

Effect of rabbit TNF on virus yield of A.) vaccinia or B.) myxoma - infected RK-13 cells. Semi-confluent monolayers in 6-well multidishes, were infected with 50 p.f u virus, VV-601 ( ), VV-MT2 (░), vMyxlac (▨) or vMyxt2g (▓). Rabbit-TNF was added at the indicated concentrations, cells harvested 72 hours post-infection, and virus titre determined by plaque assay. Points represent the mean of quadruplicate determinations, and error bars designate standard deviations.

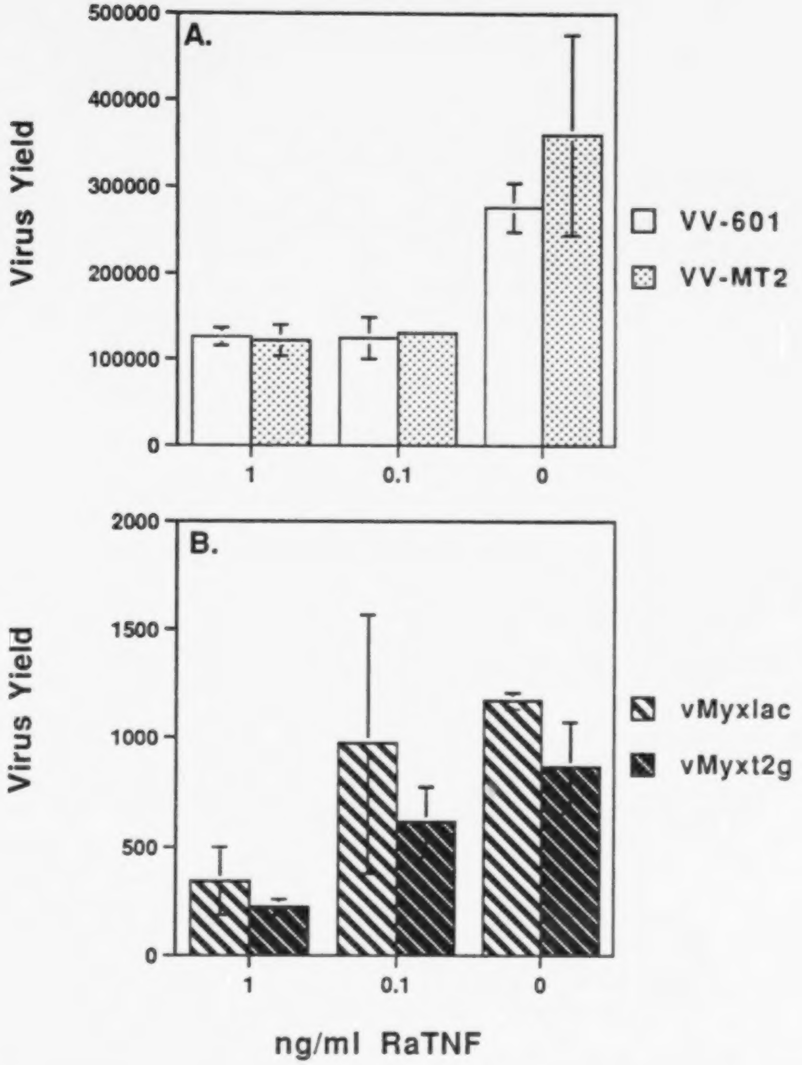

A.
Virus Yield
500000
400000
300000
200000
100000
0
1
0.1
0
VV-601
VV-MT2
B.
Virus Yield
2000
1500
1000
500
0
1
0.1
0
vMyxlac
vMyxt2g
ng/ml RaTNF

Figure III-8. Myxoma T2 Protein May Provide Protection Against the Anti-Viral Effects of Rabbit TNF

A. 24-hour virus yield from L929-8 cells co-infected at a m.o.i. of 10 with VV-MT2:VV-RaTNF or VV-601:VV-RaTNF, at a ratio of 9:1. B. 48-hour virus yield from L929-8 cells infected with VV-RaTNF or VV-601 at a m.o.i. of 0.2, or co-infected at a m.o.i. of 0.2 with VV-MT2:VV-RaTNF or VV-601:VV-RaTNF, at a ratio of 1:1. Each point represents the mean of triplicate determinations, as described under the Materials and Methods, and error bars designate standard deviations.

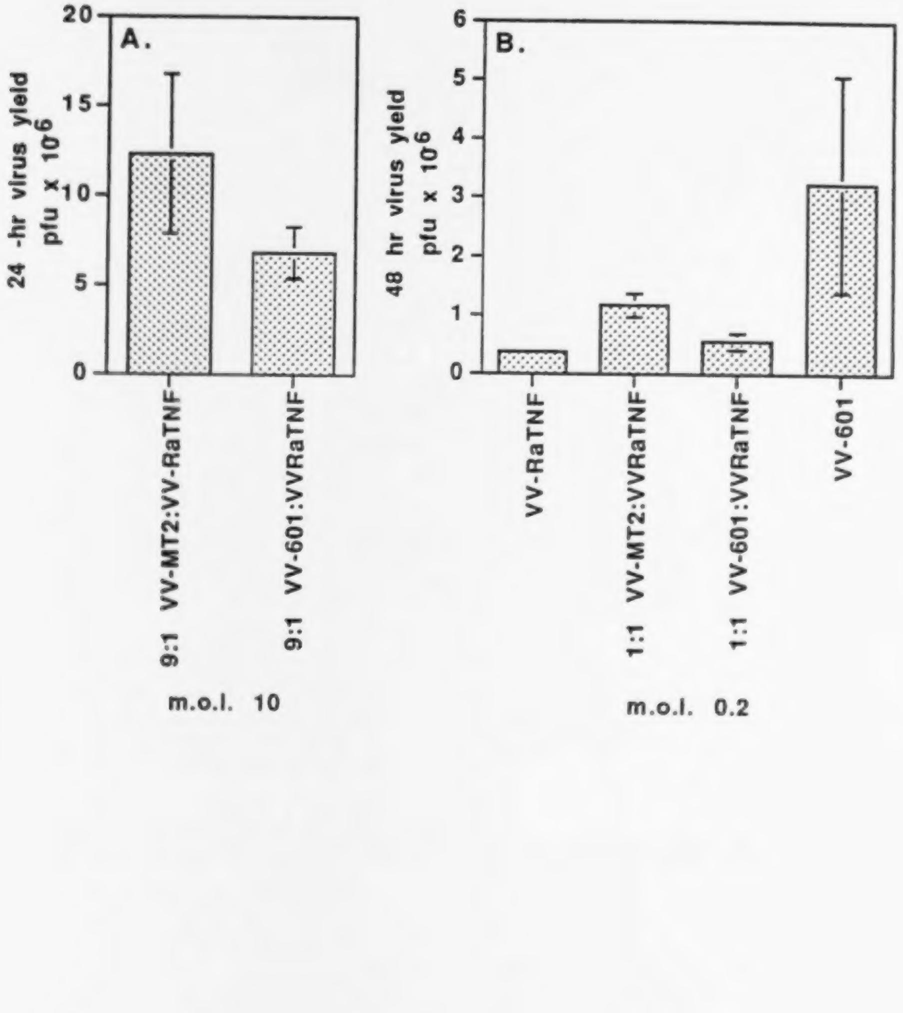

A.
24 -hr virus yield pfu x 10^6
0
5
10
15
20
9:1 VV-MT2:VV-RaTNF
9:1 VV-601:VVRaTNF
m.o.i. 10
B.
48 hr virus yield pfu x 10^6
0
1
2
3
4
5
6
VV-RaTNF
1:1 VV-MT2:VVRaTNF
1:1 VV-601:VVRaTNF
VV-601
m.o.i. 0.2

Figure III-9. Growth of Myxoma Mutants in the Presence and Absence of Rabbit TNF

RK-13 cells were infected in triplicate with vMyxlac, vMyxt2g, vMyxM11L$^-$ or vMyxSERP$^-$ at a m.o.i. of 0.1 in the presence or absence of 1 nM rabbit TNF, and the virus titers determined after 48 hours. Data is expressed as the virus yield, p.f.u. output/ p.f.u. input, the average of triplicate measurements, and error bars designate standard deviations.

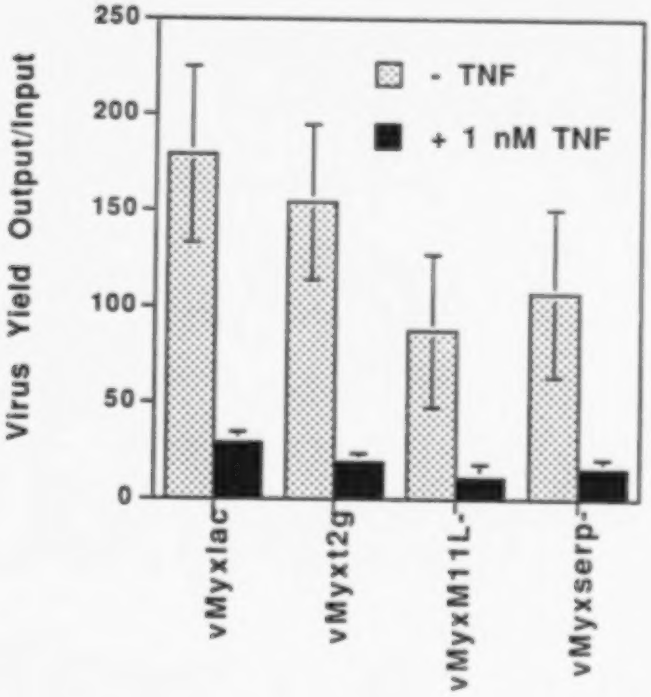

250
200
150
100
50
0
Virus Yield Output/Input
- TNF
+ 1 nM TNF
vMyxlac
vMyxt2g
vMyxM11L-
vMyxserp-

Figure III-10. Myxoma T2 Protein Protects L929-8 cells from Cytolysis by Rabbit TNF

Actinomycin D-sensitized L929-8 cells were co-incubated with various concentrations of rabbit TNF which gave a cell killing > 95%, and increasing T2 concentrations. The cell viability at different T2:TNF molar ratios (based on the mass of each monomer) was determined by neutral red uptake. Points represent the mean of triplicate determinations.

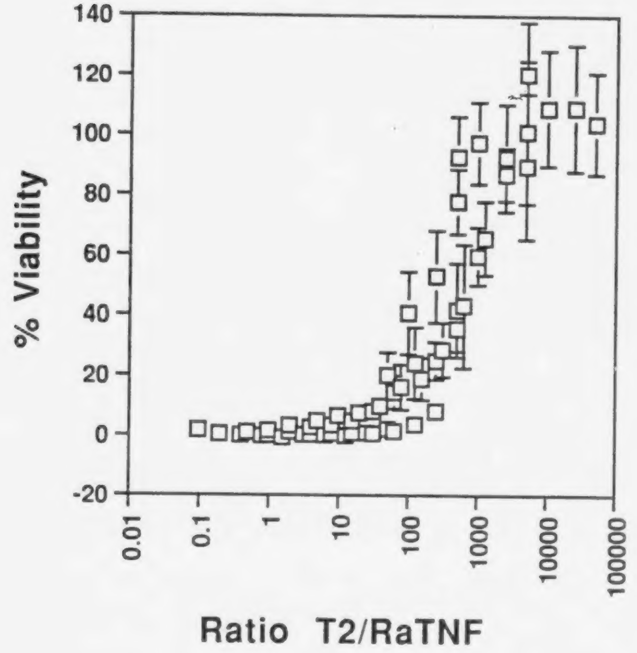

% Viability
140
120
100
80
60
40
20
0
-20
0.01
0.1
1
10
100
1000
10000
100000
Ratio T2/RaTNF

Table III-1. Myxoma T2 Protein does not Protect L929-8 Cells from Cytolysis by Mouse or Human TNF

TNF	T2:TNF Ratio	% Viability[1]	
		4 pg/mL TNF	40 pg/mL TNF
Mouse	0	11 ± 3	0
	40,000	9 ± 2	0
	20,000	11 ± 4	0
Human	0	80 ± 11	0
	40,000	72 ± 5	0
	20,000	77 ± 13	0

[1] Samples represent means of triplicate measurements

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CHAPTER IV - M-T2 PROTEIN IS SECRETED AS A MONOMER AND A DIMER AND EACH BINDS RABBIT TNF BUT THE DIMER IS A MORE POTENT TNF INHIBITOR[1]

INTRODUCTION

Biological responses to TNF are initiated when TNF or LTα bind and induce the oligomerization of cellular receptors (1, 2). Two different TNF receptors have been cloned and characterized, designated p55 TNFR and p75 TNFR, each of which binds and responds to both TNF and LTα (3–7). The TNFR superfamily, of which the M-T2 protein is a member, is characterized by the presence of multiple cysteine-rich motifs of about 40 amino acids in their extracellular amino-terminal domain (8, 9). M-T2 protein displays the greatest homology with the p75 TNFR (10), and thus rabbit TNF or LTα would appear to be the most likely candidates for a biological target of M-T2 protein, although other members of the TNF superfamily, including Fas ligand and CD40 ligand cannot be excluded.

The currently favoured model is that M-T2 protein acts to inhibit host TNF responses by binding and sequestering either extracellular or cell surface TNF and thereby preventing the activation of cellular TNF receptors. Here we demonstrate that M-T2 protein specifically forms inhibitory complexes with rabbit TNF, and that binding is associated with the ability to inhibit the cytolytic activities of rabbit TNF on susceptible rabbit and mouse cell lines. M-T2 protein is found to be secreted from virally infected cells as both a monomer and disulphide-linked dimer, both of which bind TNF with similar affinities. Moreover, the dissociation constant (K_d) values for both monomeric and dimeric M-T2 protein, assessed by Scatchard analysis, compare favourably with that of other vertebrate TNFs with their cognate receptors. However, the dimeric form of M-T2 is far more active than the monomeric form at inhibiting the cytolytic activities of TNF, most likely because as a divalent inhibitor, the dimer can more effectively inhibit the ability of the TNF trimeric ligand to induce oligermization of the cell surface TNF receptors.

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[1] A version of this chapter has been published. M. Schreiber, K. Rajarathnam and G. McFadden. 1996. *J. Biol. Chem.*, in press.

RESULTS

Purification of Myxoma T2 Protein and Rabbit TNF

Recombinant vaccinia viruses were engineered to express myxoma T2 protein (VV-MT2) and rabbit TNF (VV-RaTNF and VV-T2RaTNF) in order to obtain enough protein for binding studies (outlined in Fig. IV-1). Previously, the complete sequence for the rabbit TNF cDNA, including the 79 amino acid leader, was placed under the control of the synthetic late promoter in the recombinant virus VV-RaTNF. However, TNF was not efficiently expressed as a secreted ligand from cells infected with VV-RaTNF, so instead the recombinant vaccinia virus VV-T2RaTNF was constructed in which the presequence N-terminal to the mature rabbit TNF was excised and replaced with the 16 amino acid leader sequence of myxoma T2 protein. Pro-myxoma T2 protein was found by N-terminal sequencing to be cleaved at its predicted signal cleavage site between gly-16 and gly-17. The biologically active rabbit TNF secreted from VV-T2RaTNF infected cells has a molecular mass on SDS-polyacrylamide gels identical to the 18 kDa reported molecular mass of rabbit TNF α (11) (Fig. IV-2B).

Both myxoma T2 protein and rabbit TNF were purified from supernatants from virally-infected BGMK cells by two rounds of anion-exchange Mono Q chromatography at pH 7.5 and 6.4. Figure IV-2A displays proteins present in crude supernatants from cells infected with vector control vaccinia virus, VV601 (lane 1) and cells infected with VV-MT2 (lane 2). At pH 7.5, M-T2 protein eluted from the column at 150-280 mM NaCl (lane 3). When re-applied to the anion exchange column at pH 6.4, a single protein band was observed on silver stained SDS-PAGE gels in fractions collected at 100-150 mM NaCl, with no protein bands seen from supernatants from vector control virus, VV601, processed in identical fashion (Fig. IV-2A, lanes 4 and 5, respectively).

The purification of rabbit TNF is illustrated in Figure IV-2B, starting from supernatants of VV-T2RaTNF infected monolayers (lane 1). At pH 7.5, rabbit TNF eluted from the mono-Q column at 300-340 mM NaCl (Fig. IV-2B, lane 2). TNF-containing fractions were pooled, reapplied to the Mono-Q column at pH 6.4, and rabbit TNF eluted at 260-275 mM NaCl as a single 18 kDa species when visualized on silver stained gels (Fig 2b, lane 3).

The decreased amount of TNF secreted from VV-RaTNF-infected cells compared to VV-T2RaTNF-infected cells was not due to retention of the TNF as a 26 kDa membrane bound protein. Figure IV-2C shows rabbit TNF immunoprecipitated from supernatants and lysates of BGMK cells infected with VV-RaTNF and VV-T2RaTNF. Although 18 kDa rabbit TNF could be detected in supernatants and lysates of VV-T2RaTNF-infected cells (lanes 1 and 3), only a faint band could be detected in supernatants from VV-RaTNF-infected cells, and no 18 kDa TNF was detected in cell lysates (lanes 2 and 4). Moreover, TNF was not retained as the 26-kDa membrane bound form in VV-RaTNF-infected cells. Thus, the virus, VV-RaTNF, does not direct high level production of rabbit TNF.

The amount of M-T2 protein secreted into supernatants from BGMK cells infected with either VV-MT2 or vMyxlac virus was quantitated from densitometric scans of Western blots, using various titrations of purified M-T2 protein as standards, as described under the Materials and Methods. As expected, the amount of M-T2 protein secreted from VV-MT2 infected cells was significantly higher than from myxoma-infected cells: $(1.0\pm0.4) \times 10^8$ molecules/24 hours of M-T2 secreted per VV-MT2 infected cell compared to $(2.8\pm0.5) \times 10^6$ molecules/24 hours of M-T2 secreted per vMyxlac infected cell. In VV-MT2, the M-T2 gene is under the control of a very strong synthetic late promoter (12) which accounts for the enhanced expression compared to the standard (early) promoter in the parental myxoma virus.

Myxoma T2 Protein Specifically Complexes with Rabbit TNF

To determine whether M-T2 protein and rabbit TNF physically associate, immunoprecipitation experiments were performed. M-T2 polyclonal antibody correctly immunoprecipitates native M-T2 protein, with an apparent molecular mass of 60 kDa, from supernatants from ^{35}S met/cys labeled VV-MT2 infected cells (Fig. IV-3, lanes 4 and 10), but not from control VV-601 or VV-T2RaTNF infected cells (Fig. IV-3, lanes 5, 11, 12). Similarily, anti-rabbit TNF antibody only immunoprecipitates rabbit TNF, an 18 kDa protein from supernatants from VV-T2RaTNF infected cells (lanes 2, 8) but not from VV-601 or VV-MT2 infected cells (lanes 1, 7, 9). When supernatants from VV-601 or VV-MT2 infected cells were co-incubated with supernants from VV-T2RaTNF infected cells for 1 hour and then immunoprecipitated with anti-rabbit TNF antibody, radiolabeled proteins of sizes identical to myxoma T2 protein and rabbit TNF were co-precipitated in VV-MT2/VV-T2RaTNF mixtures (Fig. IV-3, lane 3). Similarily anti-myxoma T2 antisera was able to co-precipitate both rabbit TNF and myxoma T2 proteins from mixtures of VV-MT2/VV-T2RaTNF (lane 6). Thus, both polyclonal antisera against myxoma T2 protein and anti-rabbit TNF antibody were each able to coimmunoprecipitate the complex between rabbit TNF and myxoma T2 protein. In contrast, anti-myxoma T2 antisera was unable to co-immunoprecipitate ^{35}S-radiolabeled myxoma T2 and ^{125}I-radiolabeled mouse TNF (data not shown).

Native size of M-T2 Protein Secreted from Virus-Infected Cells

Purified T2 protein was fractionated by Superdex200 gel filtration chromatography to determine whether it is expressed as a monomer or oligomer. N-terminal sequencing had shown this to be a pure preparation of M-T2. However, as shown in Figure IV-4A, M-T2 protein eluted from the column as two distinct peaks, estimated to be 140-156 kDa and 56-65 kDa in size, based on size standards of a variety of monomeric proteins. When samples from these two peaks were analyzed by SDS-PAGE under reducing conditions and probed by Western blotting, anti-M-T2 antisera recognized only a single 55-60 kDa protein band in fractions from both peaks (Fig. IV-4A, inset), indicating that the first peak to elute from the Superdex column is most likely an oligomeric form of the second peak. When analyzed under non-reducing conditions, the first eluted M-T2 peak electrophoresed as a single 87-90 kDa band on SDS-polyacrylamide gels (Fig. IV-4B, lane 1), whereas the second M-T2 peak eluted from the Superdex column electrophoresed as a single 55-59 kDa species (lane 2). However, both M-T2 species co-migrated at approximately 60 kDa when the samples were reduced with dithiothreitol prior to electrophoresis, suggesting that the two M-T2 peaks detected by Superdex chromatography (Fig. IV-4A) are the monomeric and disulphide linked dimeric forms of the same M-T2 protein.

M-T2 protein was secreted as both a monomer and a dimer from BGMK cells and RK-13 cells infected with VV-MT2 and vMyxlac. Figure IV-4C shows a western blot of supernatants from RK-13 and BGMK cells infected with VV-MT2 and vMyxlac, probing with anti-MT2 antibody, with samples resolved in the presence and absence of dithiothreitol. In the absence of reducing agent, M-T2 protein, secreted from both BGMK and RK-13 cells infected with VV-MT2 or vMyxlac, resolved as 2 bands (Fig. IV-4C, lanes 1, 3, 5 and 7), whereas in the presence of reducing agent, M-T2 protein migrated as a single species (lanes 2, 4, 6 and 8).

To confirm this result and to resolve the discrepancies in the apparent molecular mass of M-T2 protein determined by SDS-PAGE and gel filtration chromatography, the oligomeric protein from the first Superdex 200 M-T2 peak was examined by mass spectrometry and ultracentrifugation studies. When analyzed by mass spectrometry, a mass of 80.9 kDa was calculated for the larger species, confirming that it is in fact a dimer of a 40.5 kDa species. Moreover, by ultracentrifugation sedimentation analysis, the plot of ln C vs r^2 yielded a straight line, indicating that this larger M-T2 peak from the

Superdex 200 column is a single species with a calculated molecular mass of approximately 79 kDa (Fig. IV-5).

Scatchard Analysis of M-T2 Binding to TNF

To quantitate the affinity of M-T2 protein for rabbit TNF, solid phase binding assays were performed, followed by Scatchard analysis. The Scatchard plot of a preparation of M-T2 protein containing both monomeric and dimeric forms with rabbit TNF yielded a single slope, indicative of a single affinity binding site with a K_d of 180 pM (Fig. IV-6A), but to substantiate this interpretation, binding analysis was carried out on the purified M-T2 monomer and dimer individually. The binding of ^{125}I-radiolabeled rabbit TNF with both dimeric and monomeric M-T2 protein, separated by gel filtration chromatography (Fig. IV-4A), was therefore examined using various concentrations of labeled TNF either in the presence of absence of excess unlabeled ligand. The specific binding of rabbit TNF increased with increasing concentration of labeled ligand and reached saturation at 500 pM for dimeric and monomeric M-T2 (Fig. IV-6B, C). For both forms of M-T2 the Scatchard plot of the binding data was consistent with a single class of high affinity binding sites with similar affinities, a calculated K_d of 195 pM with a correlation coefficient of -0.95 for dimeric M-T2 (Fig. IV-6B), and a K_d of 170 pM with a correlation coefficient of -0.96 for monomeric M-T2 (Fig. IV-6C). Saturable binding was also observed between M-T2 protein, containing both monomeric and dimeric species, and ^{125}I-radiolabeled mouse TNF but only at a higher concentration in the nM range (Fig. IV-7A), while no binding at all was observed with radiolabeled human TNF (Fig. IV-7B). The affinity of myxoma T2 and mouse TNF was an order of magnitude lower than the affinity of M-T2 with rabbit TNF, and the K_d was calculated to be 1.7 nM with a correlation coefficient on the Scatchard plot of -0.98. Thus, the biological specificity of M-T2 for rabbit TNF(13) is consistent with this physical binding data.

Rabbit TNF receptors have not yet been cloned or characterized. Attempts to determine the K_d of cognate rabbit TNF receptors with rabbit TNF were unsuccessful because of the low number of TNF receptors present on all rabbit cell lines screened. Instead, we tested to see whether M-T2 protein can inhibit the binding of ^{125}I-labeled rabbit TNF to cellular receptors on two rabbit cell lines that exhibited some TNF binding. Rabbit RL-5 cells, a CD4$^+$-T cell lymphoma cell line, and RK-13 cells, a fibroblast kidney line, were incubated with 500 pM ^{125}I-rabbit TNF in the presence or absence of 100-fold excess cold TNF to determine specific binding. As shown in Figure IV-8, a 400-fold molar ratio excess of purified M-T2 protein, 200 nM, was able to effectively block specific binding of rabbit TNF to both RL-5 cells (lane 2) and RK-13 cells (lane 6). Similarily, even crude supernatant from VV-MT2 infected BGMK cells, containing approximately 50 nM M-T2 protein, was also able to inhibit binding of radiolabeled rabbit TNF to RL-5 cells (lane 3), whereas control supernatant from VV-601 infected cells (lane 4) had no effect. Thus, we conclude that M-T2 protein as secreted from infected cells actually blocks the binding of rabbit TNF to the cell surface of rabbit fibroblasts and lymphocytes.

Myxoma T2 Specifically Inhibits Rabbit but not MouseTNF

In the previous chapter, we have shown that myxoma T2 protein present in crude supernatants from VV-MT2-infected cells was able to inhibit the cytolytic activities of rabbit TNF on mouse L929-8 cells, but not the mouse or human ligands. Since myxoma T2 was found by Scatchard analysis to bind both rabbit and mouse TNF albeit with different affinities (Figs. IV-6 and IV-7), anion-exchange chromatography purified myxoma T2 protein, containing both monomers and dimers, was tested for its ability to protect the TNF-hypersensitive mouse L929-8 cell line from lysis by mouse and rabbit TNF. Viability of actinomycin D-treated L929-8 cells incubated for 24 hours with various concentrations of mouse TNF was examined in the presence of increasing amounts of myxoma T2 protein, and the increase in cell viability with M-T2 present plotted against the molar ratio of myxoma T2:mouse TNF. As shown in Figure IV-9A, myxoma T2 protein did not engender enhanced viability of L929-8 cells even at M-T2:mouse TNF ratios approaching 50,000 to 1. However, M-T2 protein, containing a mixture of both monomeric and dimeric forms, was able to protect L929-8 cells from lysis by rabbit TNF at ratios of 10:1 for M-T2:rabbit TNF (Fig. IV-9B) with complete protection against rabbit TNF seen at ratios of 400:1.

Although rabbit TNF is active on mouse cells, it is of relevance to assess the more biologically relevant interaction with rabbit cells. The ability of myxoma T2 protein to protect rabbit RK-13 cells from lysis by rabbit TNF was also tested. Actinomycin-D sensitized RK-13 cells are not as susceptible to TNF cytolysis as are L929-8 cells and evidence a maximal loss of viability down to 30% of untreated cultures even at the highest rabbit TNF concentrations tested. When exposed to rabbit TNF concentrations at which cytolysis was maximal, myxoma T2 protein was able to effectively protect RK-13 cells against lysis by rabbit TNF, with complete protection observed at M-T2:rabbit TNF monomer molar ratios of 50:1. (Fig. IV-9C).

Dimeric M-T2 Protein is Much More Effective than the Monomer at Inhibiting the Cytolytic Activity of Rabbit TNF

To determine whether the monomeric and dimeric forms of M-T2 are comparably active at inhibiting the biological activities of rabbit TNF, actinomycin D-sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF, in the presence of 5 nM dimeric T2 or 10 nM monomeric T2. As shown in Figure IV-10A, a 5-fold molar excess of dimeric M-T2 protein (solid squares) can protect against TNF-induced lysis, with complete protection detected by 50-fold excess. However, 10 nM monomeric M-T2 (triangles) was completely unable to protect L929-8 from lysis by rabbit TNF compared to controls (circles). Only when present at much higher concentrations was monomeric M-T2 protein able to protect against TNF-induced cytolysis, in that 80% protection was observed at a 2000-fold molar excess monomeric M-T2 protein (triangles, Fig. IV-10B).

Interestingly, comparable levels of rabbit TNF were able to co-immunoprecipitate with both the M-T2 monomer and dimer. When [^{35}S]-met/cys labeled rabbit TNF was co-incubated with either monomeric or dimeric M-T2 protein, and then immunoprecipitated with anti-M-T2 antisera (Fig. IV-10C), both monomeric and dimeric M-T2 protein co-immunoprecipitated similar amounts of rabbit TNF (lanes 3, 4), indicating that both forms of M-T2 protein form stable complexes with rabbit TNF. We conclude that dimeric M-T2, with two ligand binding sites, can likely occlude two of the three TNF receptor binding domains present in a TNF trimer, and is therefore a much more effective inhibitor of TNF than is monomeric M-T2.

DISCUSSION

Myxoma virus, a member of the poxvirus family of viruses, encodes a wide spectrum of immunomodulatory proteins, including several secreted homologs of cytokine receptors (14, 15). M-T2 protein, which is encoded by two copies of the T2 gene, possesses homology to the extracellular ligand binding domains of the TNFRs and presumably acts as a TNF antagonist, binding and sequestering host TNF thus preventing this ligand from participating in the local antiviral response (10). In the previous chapter, we have shown that M-T2 protein inhibits the cytolytic activity of rabbit TNF on mouse L929-8 cells, but not mouse or human TNF (13). Here we show that M-T2 and rabbit TNF form stable inhibitory complexes and that the dimeric form of M-T2 is the superior inhibitor of TNF biological activity.

Biochemical characterization of M-T2 protein revealed that it is secreted from virus infected cells as both a 40 kDa monomer and a 80 kDa disulphide-linked dimer. Because of discrepancies in the apparent molecular weights between SDS-PAGE and gel filtration analysis, we used mass spectrometry and ultracentrifugation sedimentation equilibrium techniques to confirm the size and oligomeric status of M-T2. M-T2 protein was detected as both a monomer, approximately 40 kDa in size, and an 80.9 kDa dimer, as determined by mass spectrometry. Moreover, sedimentation equilibrium analysis of the dimer indicated that it was a single stable species, of approximately 79 kDa. The calculated molecular mass from amino acid composition of unmodified, monomeric, secreted M-T2 is predicted to be 33 kDa. The difference between the theoretical and observed molecular masses may be accounted for by N-linked glycosylation at one or more of the four predicted glycosylation sites on the M-T2 protein (unpublished results).

Both the monomeric and dimeric forms of M-T2 protein form stable complexes with rabbit TNF with similar binding affinities. High affinity binding with K_ds of 170 pM and 195 pM were observed between rabbit TNF and monomeric and dimeric M-T2, respectively, by solid phase binding and Scatchard analysis. Because of the low number of TNFRs present on rabbit cell lines tested, we were unable to accurately quantitate the affinity of rabbit TNF for rabbit cell surface TNFRs, but reported K_ds for the binding of human and mouse TNF to the p75 TNFR range from 50-500 pM (4, 16-18),whereas the p55 TNFR displays a somewhat lower affinity with reported K_ds ranging from 130-2300 pM (18-20). The calculated K_d for myxoma T2 protein with rabbit TNF thus compares favourably with the K_ds determined for the human and mouse TNFRs with their cognate TNFs.

Soluble TNFRs, consisting of truncated versions of the extracellular ligand binding domains of the p55 and the p75 receptors, bind TNF with an affinity comparable to the full-length membrane receptors (16, 19, 21). This is in contrast to many soluble receptors, including the IFN-γ receptor, which often bind their respective ligands with lower affinity than the membrane-bound counterpart (22, 23). The myxoma virus homolog of the IFN-γ receptor (M-T7), proposed to act in an analogous fashion to M-T2, binds rabbit IFN-γ with a K_d of 1.2 nM (24), which is 6-7-fold lower affinity than M-T2 for rabbit TNF. However, M-T7 is by far the most abundantly secreted viral protein from myxoma infected cells (25), with 4 x 10^7molecules secreted per infected cell in the first few hours of myxoma infection (24), whereas only 3 x 10^6 molecules of M-T2 are secreted per infected cell over the course of a single infection cycle. Moreover a wide variety in the number of TNF receptors present on various cell types has been reported, ranging from 100-10,000 copies (4, 26, 27), which may be accounted for by a low, constitutive expression of p55 TNFR (1000 copies/cell) and an inducible level of p75 TNFR expression (4, 27). It is the p55 TNFR which is believed to signal the vast majority of the pathogen response activities of TNF (5, 6) including cytotoxicity (7, 28) and antiviral activity (29). Thus we presume that M-T2 must block ligand interaction with the presumptive rabbit version of the p55 TNFR to inhibit the direct cytolytic or antiviral activities of TNF. The level of M-T2 protein secreted from infected cells is still in vast excess over the estimated number of p55 TNFRs present on TNF responsive cells, and levels of M-T2 secreted by myxoma virus should therefore be sufficient to inhibit TNF within the microenvironment of virus-infected tissues.

Full length p55 receptor has an equivalent affinity for both TNF and LTα while the soluble version of the same receptor displays a 50-60 fold lower affinity for LTα (21, 30). A comparison of M-T2 affinity for rabbit TNF and LTα would indicate whether LTα is also a candidate for M-T2 inhibitory action as well. However, although the rabbit TNF gene locus has been sequenced, and the amino acid sequence of rabbit LTα deduced from the genomic sequence (31), all our attempts at isolating rabbit LTα cDNA using this sequence were unsuccessful, and thus we cannot at this point comment on whether rabbit LTα is a biologically relevant target for M-T2 in addition to rabbit TNF.

Although solid phase saturable binding between mouse TNF and myxoma T2 protein was measured (K_d of 1.7 nM), we were unable to co-immunoprecipitate M-T2 protein complexed with mouse TNF (data not shown). Moreover, purified myxoma T2 protein was unable to protect mouse L929-8 cells from lysis by mouse TNF even at M-T2:TNF molar ratios of 50,000:1, confirming our earlier observations on the species specificity of M-T2, and further re-inforcing the idea that binding and inhibition are not synonymous for this class of viral cytokine inhibitors.

On the other hand, purified M-T2 protein was able to effectively protect mouse and rabbit cells from lysis by rabbit TNF, with 50% protection observed at 50-fold molar excess of M-T2 protein, and complete protection observed at 300-fold molar excess of M-T2. M-T2 protein was even more effective at protecting rabbit RK-13 cells from lysis by rabbit TNF, in that a 50% inhibition of TNF cytotoxicity was observed at only a 10-fold molar excess of M-T2, and complete protection seen at 50-fold excess. Thus, M-T2 protein is able to prevent rabbit TNF signaling not only through heterologous mouse TNFRs but also to the homologous rabbit TNFRs. One might predict that rabbit TNF receptors would have a higher affinity for rabbit TNF than do the mouse receptors, and that M-T2 protein would be better at competing with mouse TNFRs than rabbit receptors for binding rabbit TNF. However, it should be cautioned that L929-8 cells were selected for their hypersensitivity to TNF (including the mouse, human and rabbit ligand), and the increased amounts of M-T2 protein required for protection may simply reflect a heightened capacity of L929-8 cells to effect a TNF signaling cascade with relatively fewer molecules of TNF binding to surface receptors.

A 10 to 500-fold molar excess of human p55 soluble TNFR is required to inhibit the cytolytic activity of human TNF on various cell lines (19, 21, 32, 33), which compares favourably with the ratios of M-T2 required to inhibit rabbit TNF. In comparing the ability of the human soluble p55 and p75 receptors to inhibit the cytolytic effects of human TNF on L929-8 cells, Hale *et al* (1995) found that the soluble p55 receptor was a 10-fold more potent inhibitor than the soluble p75 receptor (32). Moreover, the soluble p55 receptor was 100-fold more effective than soluble p75 receptor at blocking binding of TNF to U-937 cells (34). The kinetics of association and dissociation of TNF with the p75 TNFR is much more rapid than with the p55 TNFR (35), which may account for its reduced effectiveness as a TNF inhibitor. Although M-T2 and the related S-T2 protein from Shope fibroma virus show greater sequence similarity with the p75 TNFR than the p55 TNFR (36), we show here that M-T2 protein is as potent an inhibitor of TNF cytolysis as is the soluble p55 TNFR.

According to our model of M-T2 action, M-T2 sequesters TNF into stable inhibitory complexes and prevents its association with receptors present on the cell surface. However, another possibility is that M-T2 binding of TNF may not necessarily occlude binding of the TNF trimer to cellular receptors, but may prevent receptor bound TNF from signaling, perhaps by preventing the subsequent clustering of membrane TNF receptors. However, we have shown that a 400-fold molar excess of M-T2 was able to effectively block binding of ^{125}I-labeled rabbit TNF to both rabbit lymphocytes and fibroblasts to near background levels. At this molar ratio of M-T2:TNF, RK-13 fibroblast cells show complete protection against lysis by TNF although the same experiment could not be performed with RL-5 lymphocytes because they are not sensitive to TNF cytolysis (M. Schreiber and G. McFadden, unpublished). As the protective capabilities of M-T2 correlate with its ability to inhibit binding of TNF to membrane receptors, we conclude that M-T2 inhibits the cytolytic activities of TNF by physically preventing TNF from associating with its cognate TNF membrane receptors.

Although both the monomeric and dimeric species of M-T2 bind rabbit TNF with similar affinities, the dimeric form of M-T2 was much more effective at inhibiting cytolysis of L929-8 cells by rabbit TNF than the monomeric form. A 25-fold molar excess of dimeric M-T2 protein, compared to a 2000-fold excess of monomeric M-T2 protein, was required to completely inhibit the cytolytic activity of rabbit TNF on L929-8 cells. Similar observations have been made with monomeric and dimeric forms of the cellular soluble TNFRs. Dimeric soluble TNFRs, consisting of chimeric immunofusion proteins of the extracellular ligand binding domains with human IgG Fc, are much more active at inhibiting the biological activities of TNF than the corresponding soluble receptors (19, 22, 33, 37-39). Concentrations of dimeric soluble p55 TNFR equimolar to TNF are sufficient to neutralize the cytolytic activity of TNF on WEHI-164, SK MEL

109, and KYM-1D4 cells, whereas a 10 to 1000-fold excess of soluble p55 TNFR monomer was required to achieve similar levels of inhibition (19, 40, 41). Part of the enhanced inhibitory activity of the bivalent immunofusion proteins can be accounted for by their higher affinity for TNF than the monovalent soluble TNFRs (19, 22, 38), which most likely reflects an increased avidity of the bivalent construct for its ligand. However, the difference between the dimeric constructs and soluble receptors in inhibiting TNF activity (10-1000 fold) is significantly greater than their differences in binding affinity for TNF (6-8 fold). Clustering of membrane bound TNFRs, either by dimerization or trimerization, is believed to be important in establishing a downstream signal (1, 2, 4). Dimerization of erythropoietin/p55 or p75 TNFR chimeras is sufficient to to elicit a biological response (42, 43), although receptor dimerization may be less effective at initiating signal transduction cascades than trimerization (4, 43). Dimeric soluble TNFRs could simultaneously block two receptor binding sites on a single TNF trimer, thus rendering TNF unable to even dimerize cell surface TNF receptors (37), whereas two soluble TNFRs would have to bind a TNF trimer in order to similarily inactivate it from dimerizing receptors. Dimeric M-T2 protein thus could act in an analogous fashion, by preventing even dimerization of cellular TNFRs, which would account for its 100-fold greater inhibitory activity over that of the monomeric M-T2 protein. Thus, we conclude that poxviruses exploit the greater inhibitory capacity of linked receptor dimers, thus strengthening the concept that the effective blockade of cytokine function can be achieved by sequestering important ligands such as TNF into inhibitory complexes that disallow receptor oligomerization and subsequent intracellular signaling cascades.

Figure IV-1. Structure of Recombinant Vaccinia Viruses Expressing M-T2 and Rabbit TNF

Structure of VV-601, VV-MT2, VV-RaTNF and VV-T2RaTNF in which M-T2 sequence, the entire rabbit TNF coding sequence, or the MT2 leader sequence/mature rabbit TNF coding sequence were placed under the control of the vaccinia synthetic late promoter (SLP), bent arrow, and inserted together with *E. coli* lacZ into the *tk* locus of vaccinia-WR. Asterisks indicate predicted N-linked glycosylation sites, oval boxes designate the four cysteine-rich repeats of M-T2 and arrowheads designate predicted N-terminal cleavage sites, TIR designates the terminal inverted repeats.

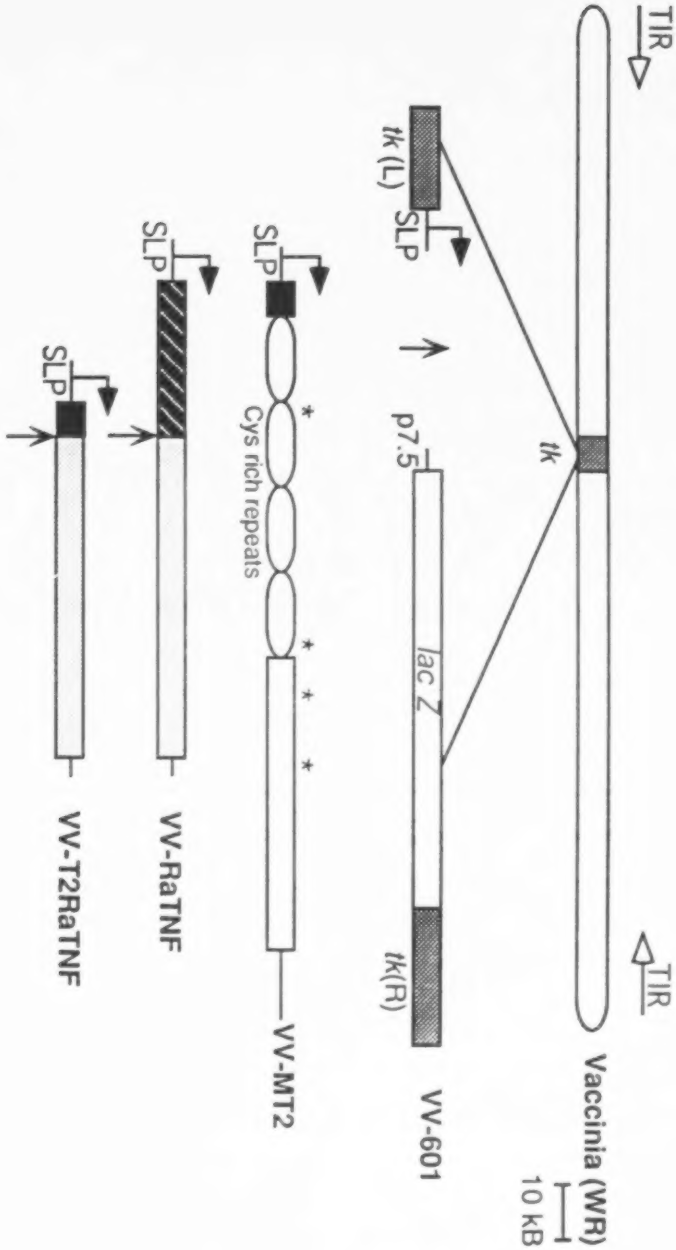

TIR
TIR
Vaccinia (WR)
10 kB
tk
tk (L)
SLP
p7.5
lac Z
tk(R)
VV-601
SLP
Cys rich repeats
VV-MT2
SLP
VV-RaTNF
SLP
VV-T2RaTNF

Figure IV-2. Purification of MT2 and Rabbit TNF Proteins and Immunoprecipitation of Rabbit TNF from VV-T2RaTNF and VV-RaTNF-Infected Cells

A. Purification of M-T2 displaying proteins present in crude supernatants from VV-601 (lane 1) or VV-MT2 infected cells (lane 2), purity of MT2 after the first anion-exchange chromatography, pH7.5 (lane 3), followed by a second anion exchange chromatography pH 6.4 (lane 4), and corresponding fraction from control VV-601 supernatants (lane 5).

B. Purification of secreted rabbit TNF from crude supernatants from VV-T2RaTNF infected cells (lane 1), and purity of rabbit TNF after the first anion-exchange chromatography pH 7.5 (lane 2) followed by a second anion exchange crhomatography pH 6.4 (lane 3). Size standards of 66.2, 45, 31 21.5 and 14.4 kDa are marked on panels A and B.

C. Immunoprecipitation of ^{35}S-labeled rabbit TNF from supernatants (lanes 1 and 2) and lysates (lanes 3 and 4) of BGMK cells infected with VV-T2RaTNF (lanes 1 and 3) and VV-RaTNF (lanes 2 and 4), using an anti-rabbit TNF antibody, and cells radiolabeled 6-22 hours post infection, as described under the Materials and Methods. Size standards of 84, 53.2, 34.9, 28.7 and 20.5 kDa are indicated.

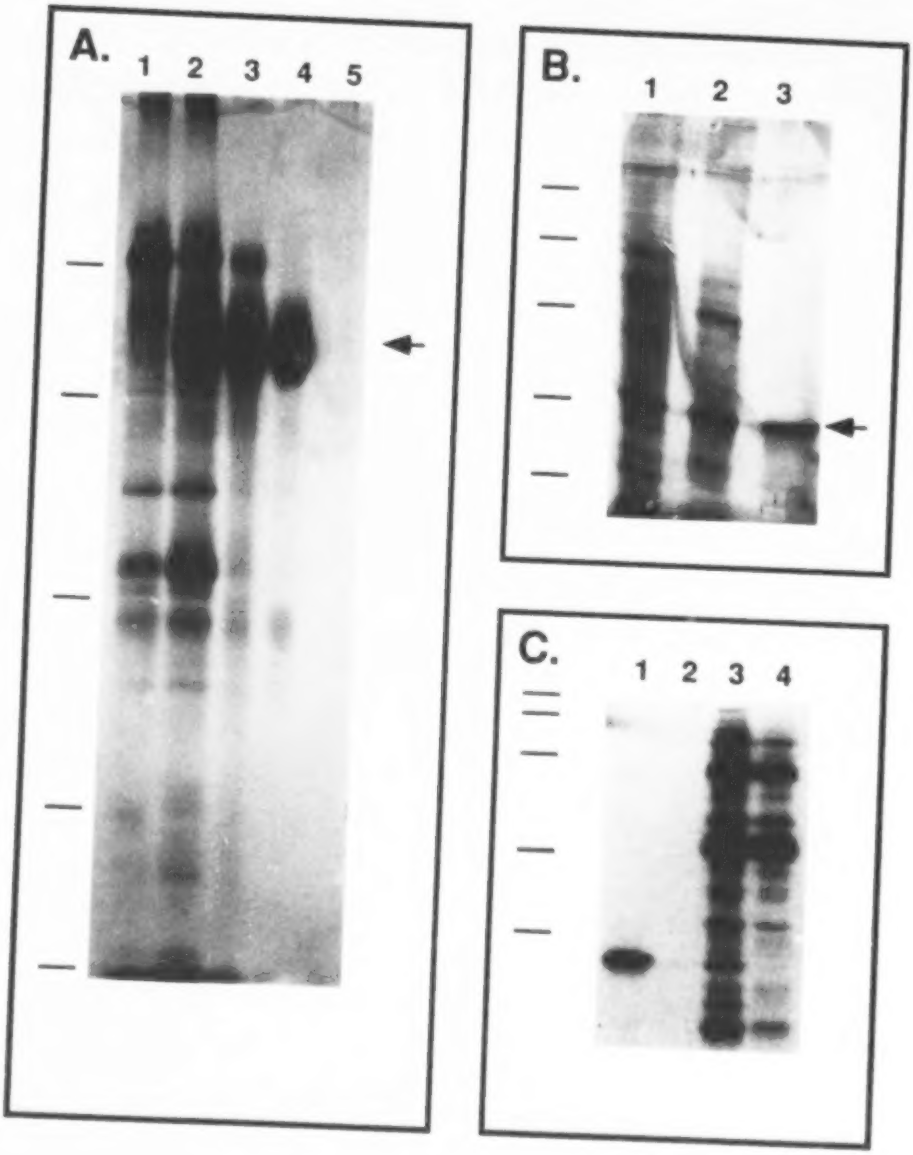

A.
1
2
3
4
5
B.
1
2
3
C.
1
2
3
4

Figure IV-3. Co-immunoprecipitations of MT2 and Rabbit TNF

^{35}S-labeled proteins from VV-T2RaTNF infected cells (lanes 2,3,5,6,8 and 10), VV-MT2 infected cells (lanes 1,3,4,6,9 and 10) or control VV-601-infected cells (1,2,4,5,7,11), were combined in pairs (lanes 1-6), or singly (lanes 7-12), and immunoprecipitated with anti-rabbit TNF antibody (lanes 1-3,7-9) or polyclonal rabbit anti-MT2 antisera (lanes 4-6, 10-12) as described under "Materials and Methods" and analyzed by SDS-PAGE and autoradiography. Size standards of 112, 84, 53.2, 34.9, 28.7 and 20.5 kDa are indicated.

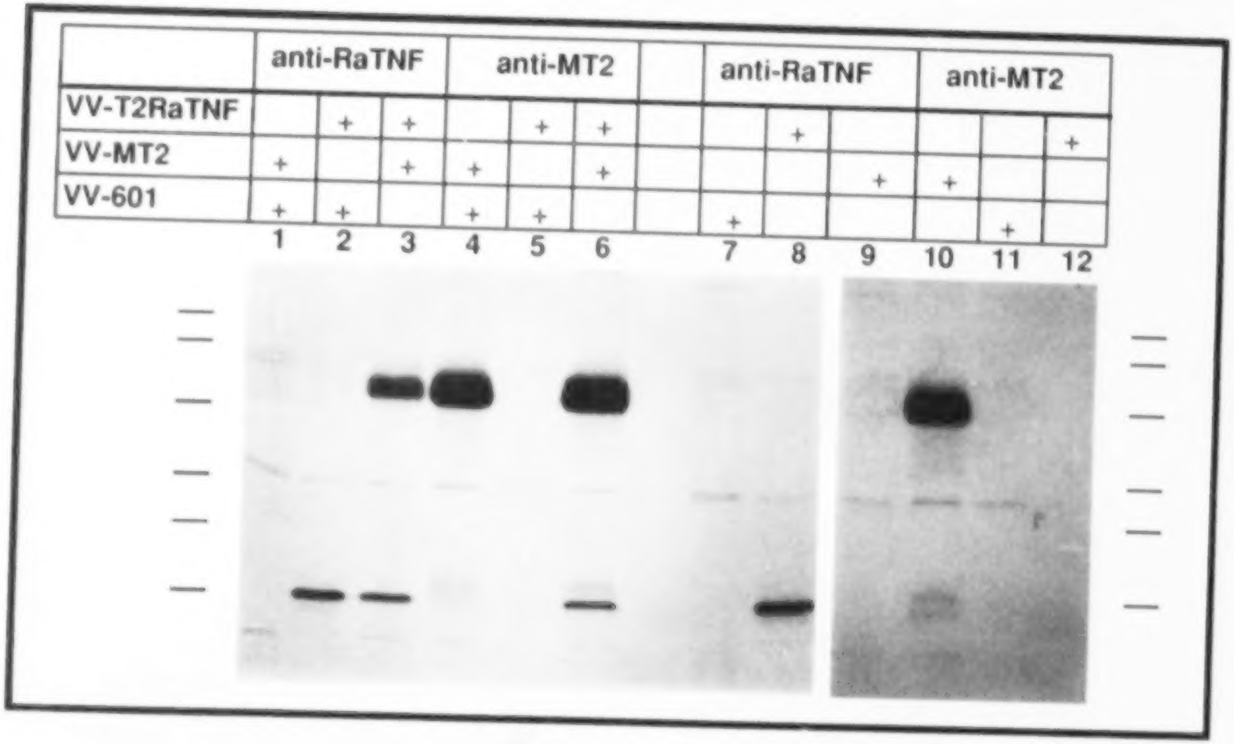


Figure IV-4. Native Size of M-T2 Protein

A. Elution profile of purified M-T2 protein fractionated by Superdex 200 gel filtration chromatography, showing protein concentration, O.D.$_{280}$ (triangles) as a function of fraction number. Inset shows a Western blot of a reducing SDS-PAGE gel testing various fractions, probed with a rabbit anti-MT2 antiserum. Elution of size standards bovine serum albumin, 67 kDa; aldolase, 158; catalase, 232; ferritin, 440; and thyroglobulin, 669 kDa (circles) are shown as a function of the log molecular weight.

B. Western blot of protein present in fraction 160 (lanes 1, 3) and fraction 192 (lanes 2, 4) run under non-reducing (lanes 1,2) and reducing (3,4) conditions, and probed with rabbit-anti MT2 antisera as outlined under "Materials and Methods". D, dimer; M, monomer.

C. Western blot of proteins secreted from RK-13 (lanes 1, 2, 5, and 6) and BGMK cells (lanes 3, 4, 7, and 8) infected with VV-MT2 (lanes 1-4) and vMyxlac (lanes 5-8), resolved under non-reducing (lanes 1, 3, 5, 7) and reducing conditions (lanes 2, 4, 6 and 8), and probed with rabbit-anti MT2 antisera as described under the Materials and Methods. Supernatants were harvested from cells infected at a m.o.i. of 10 for 24 hours; vMyxlac-supernatants were concentrated 10-fold with Ultrafree-MC 10,000 NMWL filter units before analysis by SDS-PAGE.

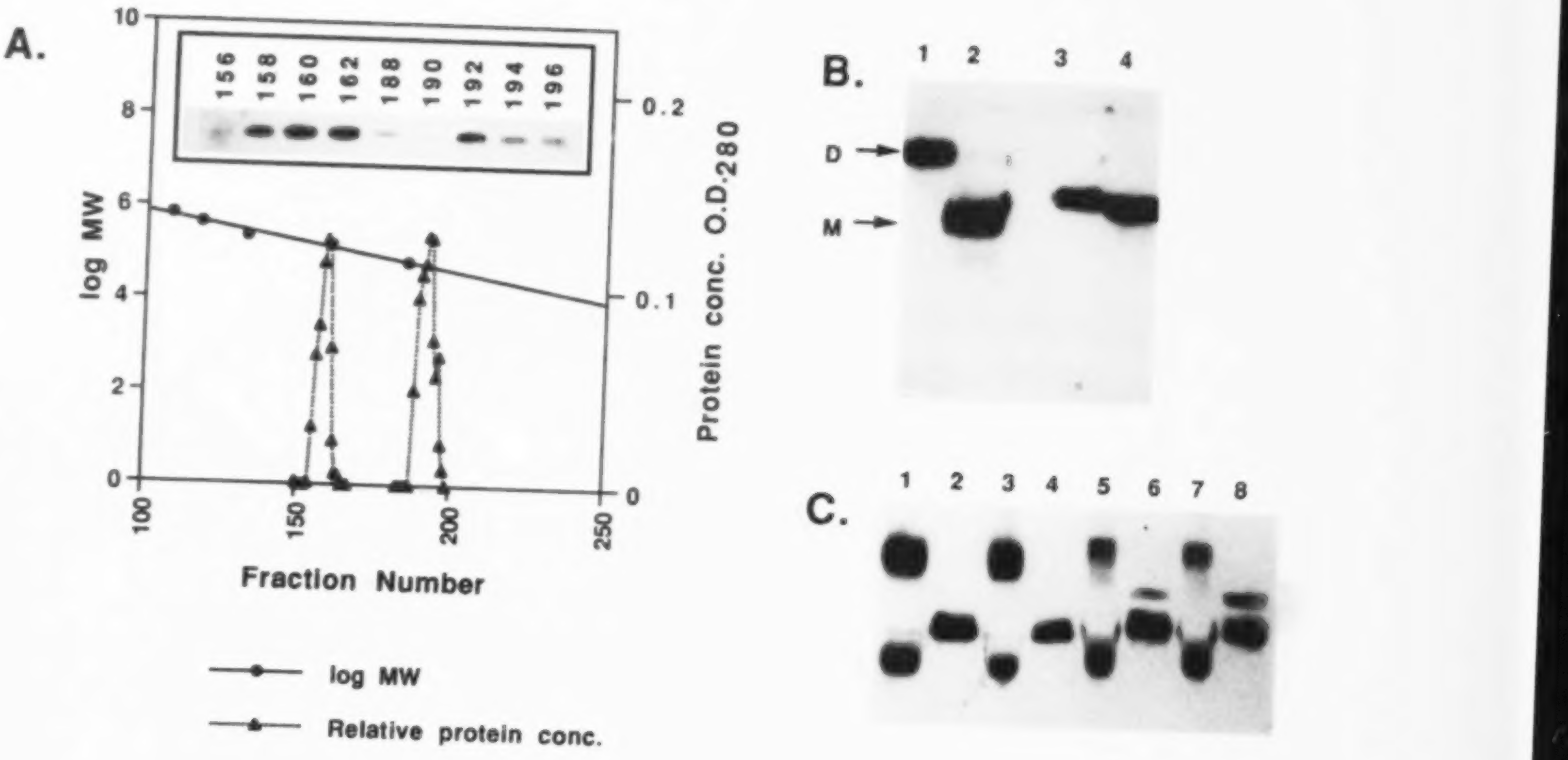

A.
10
8
6
4
2
0
log MW
156
158
160
162
188
190
192
194
196
0.2
0.1
0
Protein conc. O.D.280
100
150
200
250
Fraction Number
log MW
Relative protein conc.
B.
1
2
3
4
D
M
C.
1
2
3
4
5
6
7
8

Figure. IV-5 Ultracentrifugation Analysis of M-T2 Protein

Analysis of the sedimentation equilibrium run performed as described under "Materials and Methods", showing the plot of ln$C$ versus r^2, where C is the concentration in absorbance units at 280 nm and r is the distance from the axis of rotation (cm). The molecular weight of ~79 kDa was calculated from the slope using the equation given under "Materials and Methods.

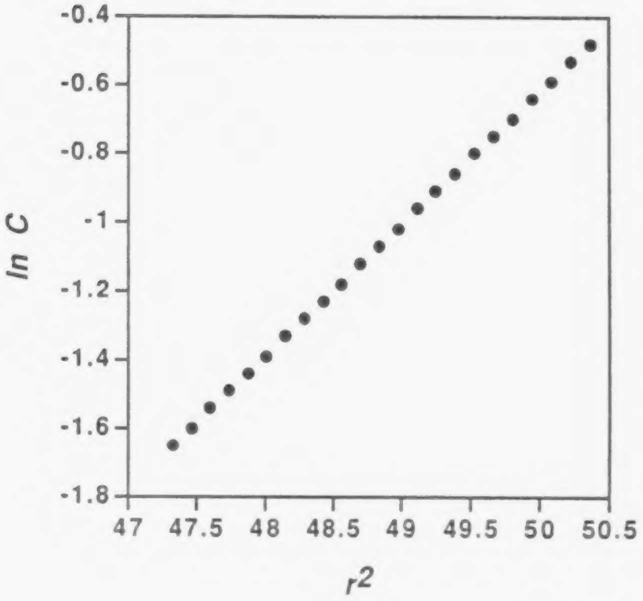

-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
-1.8
ln C
47
47.5
48
48.5
49
49.5
50
50.5
r^2

Figure IV-6. Solid Phase Equilibrium Binding Analysis of ^{125}I-labeled Rabbit TNF to M-T2 Protein

Solid phase analysis of (A) anion-exchange chromatography purified M-T2 protein containing a mixture of monomers and dimers (B) dimeric M-T2 protein and (C) monomeric M-T2 protein with ^{125}I-labeled rabbit TNF, and Scatchard analysis (insets) of the binding curves of ^{125}I-labeled rabbit TNF and MT2, as outlined under "Materials and Methods".

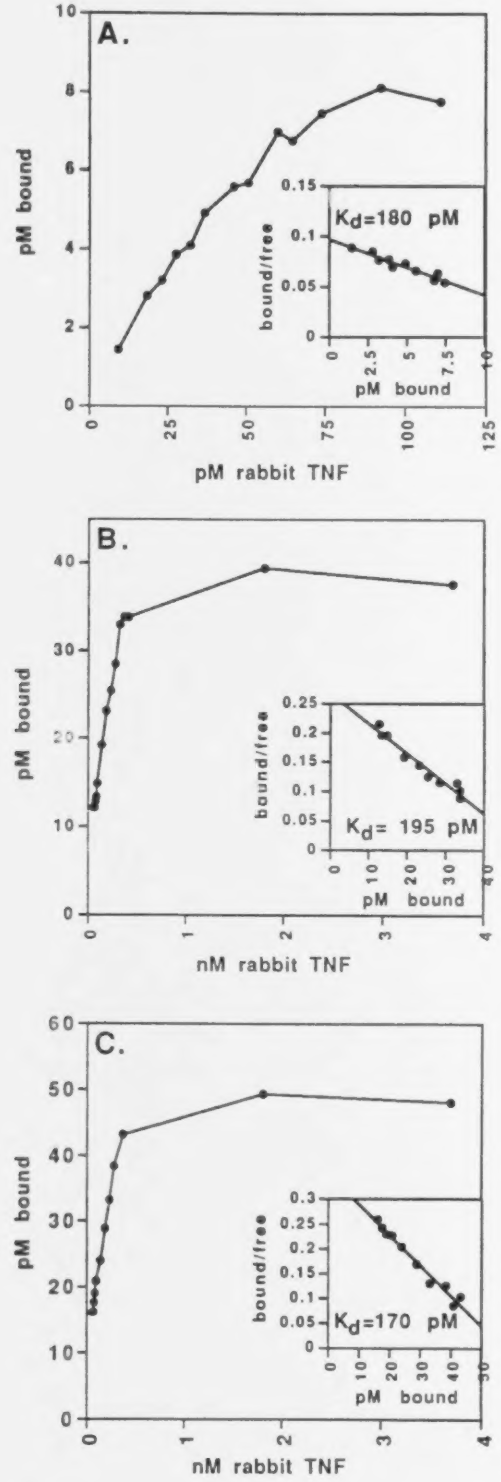

A.
pM bound
pM rabbit TNF
0
2
4
6
8
10
0
25
50
75
100
125
Kd=180 pM
bound/free
pM bound
0
0.05
0.1
0.15
0
2.5
5
7.5
10
B.
pM bound
nM rabbit TNF
0
10
20
30
40
0
1
2
3
4
Kd= 195 pM
bound/free
pM bound
0
0.05
0.1
0.15
0.2
0.25
0
10
20
30
40
C.
pM bound
nM rabbit TNF
0
10
20
30
40
50
60
0
1
2
3
4
Kd=170 pM
bound/free
pM bound
0
0.05
0.1
0.15
0.2
0.25
0.3
0
10
20
30
40
50

Figure IV-7. Solid Phase Equilibrium Binding Analysis of Mouse and Human ^{125}I-labeled TNF to M-T2 Protein

A. Solid phase analysis of MT2 protein with ^{125}I-labeled mouse TNF, and Scatchard analysis (inset) of the binding curve of ^{125}I-labeled mouse TNF and MT2.

B. Solid phase analysis of MT2 protein with ^{125}I-labeled human TNF.

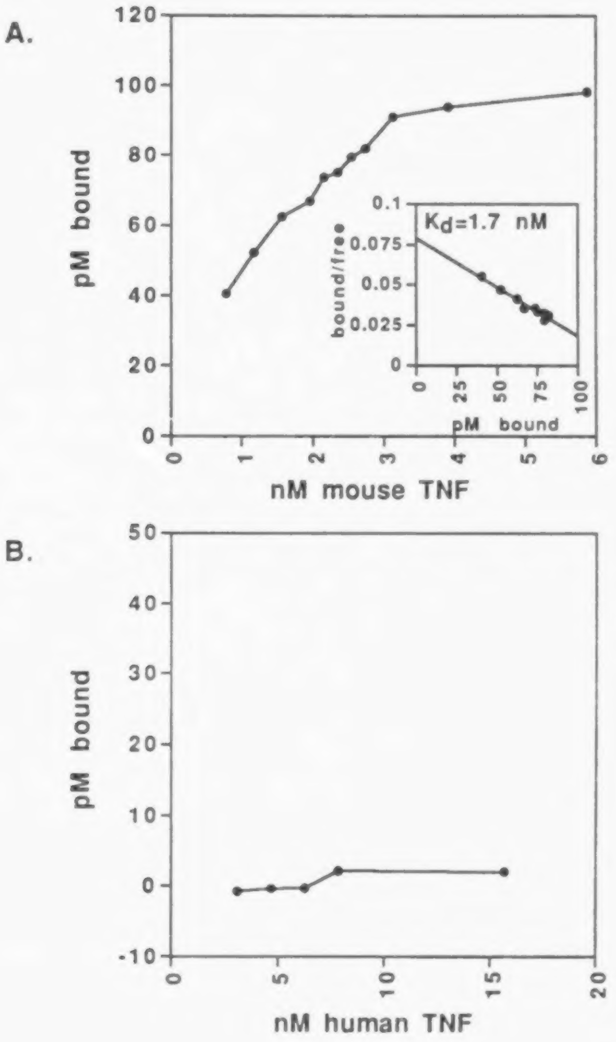

A.
120
100
80
60
40
20
0
pM bound
0
1
2
3
4
5
6
nM mouse TNF
0.1
0.075
0.05
0.025
0
bound/free
Kd=1.7 nM
0
25
50
75
100
pM bound
B.
50
40
30
20
10
0
-10
pM bound
0
5
10
15
20
nM human TNF

Figure IV-8. M-T2 Inhibition of the Binding of Rabbit TNF to Rabbit Membrane TNFRs

2 x 10^6 RL-5 cells (speckled box) or 1 x 10^6 RK-13 cells (open box) were incubated with 500 pM ^{125}I-labeled rabbit TNF in the presence and absence of 100-fold excess cold TNF to determine ligand specific binding to the cell surface. 200 nM purified M-T2 protein, or 10% vol/vol supernatant from VV-601 infected or VV-MT2 infected cells (containing approximately 50 nM MT2 protein) were added as indicated, as outlined under "Materials and Methods". Standard deviations of triplicate assays are shown.

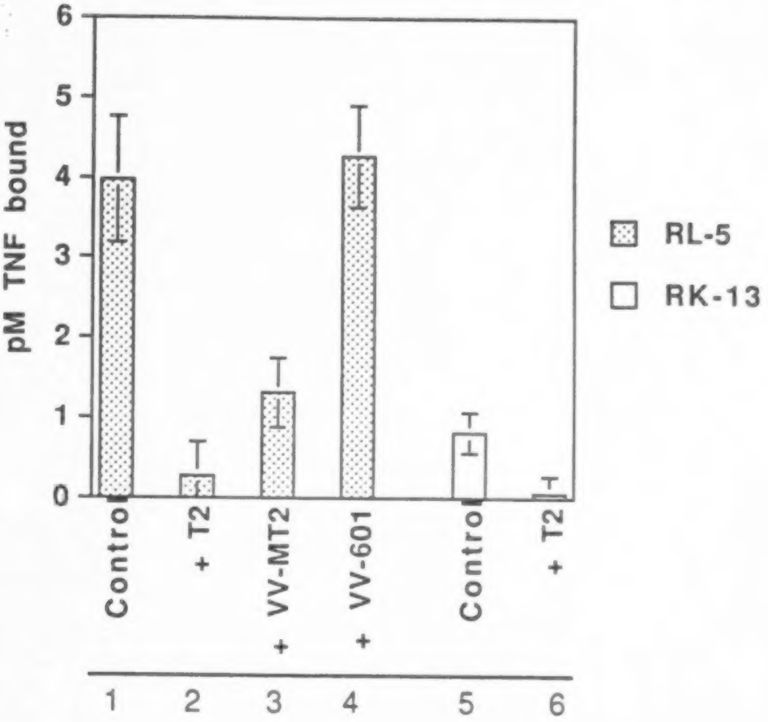

pM TNF bound
0
1
2
3
4
5
6
RL-5
RK-13
Control
+ T2
+ VV-MT2
+ VV-601
Control
+ T2
1
2
3
4
5
6

Figure. IV-9. M-T2 Protects Against Cytolysis by Rabbit but not Mouse TNF

Actinomycin- D sensitized L929-8 cells (A, B) or RK-13 cells (C) were incubated at varying molar ratios of M-T2 protein and mouse TNF (A) or rabbit TNF (B,C) as described under "Materials and Methods" and the % increase in cell viability (a) or % viability of cells (B,C) determined by neutral red uptake (A,B) or crystal violet staining (C). A monomeric size of 40 kDa was calculated for M-T2 while the trimeric size of 54 kDa for rabbit TNF and 51 kDa for mouse TNF was used. Points represent the mean of quadruplicate determinations.

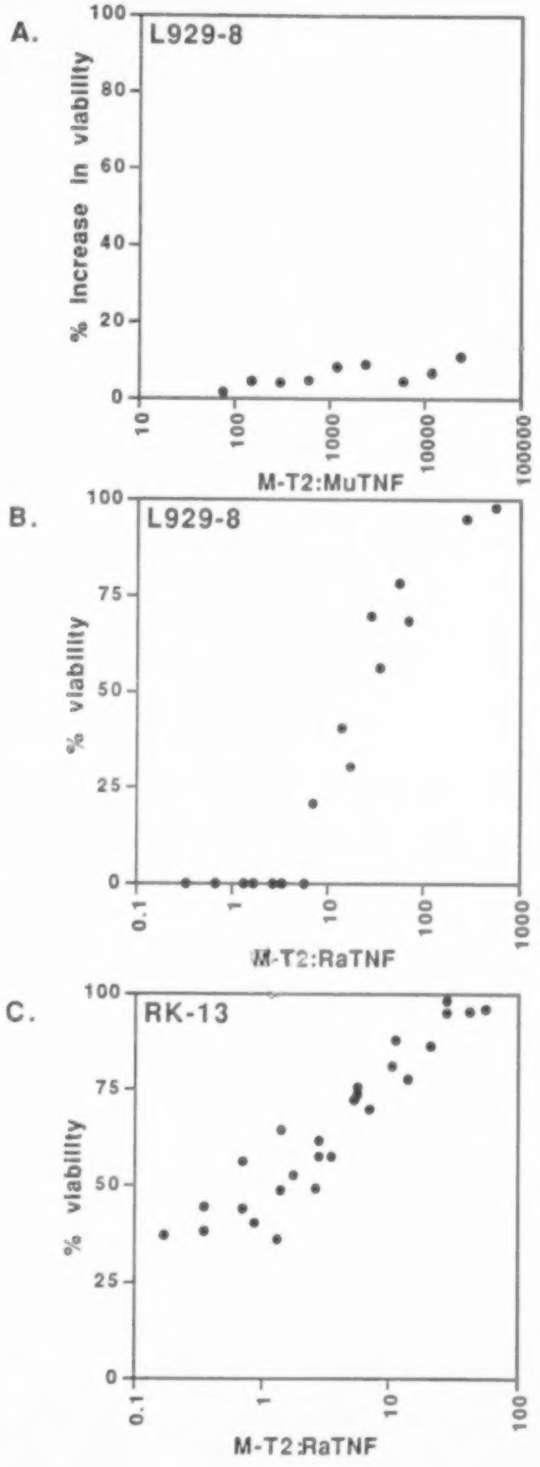
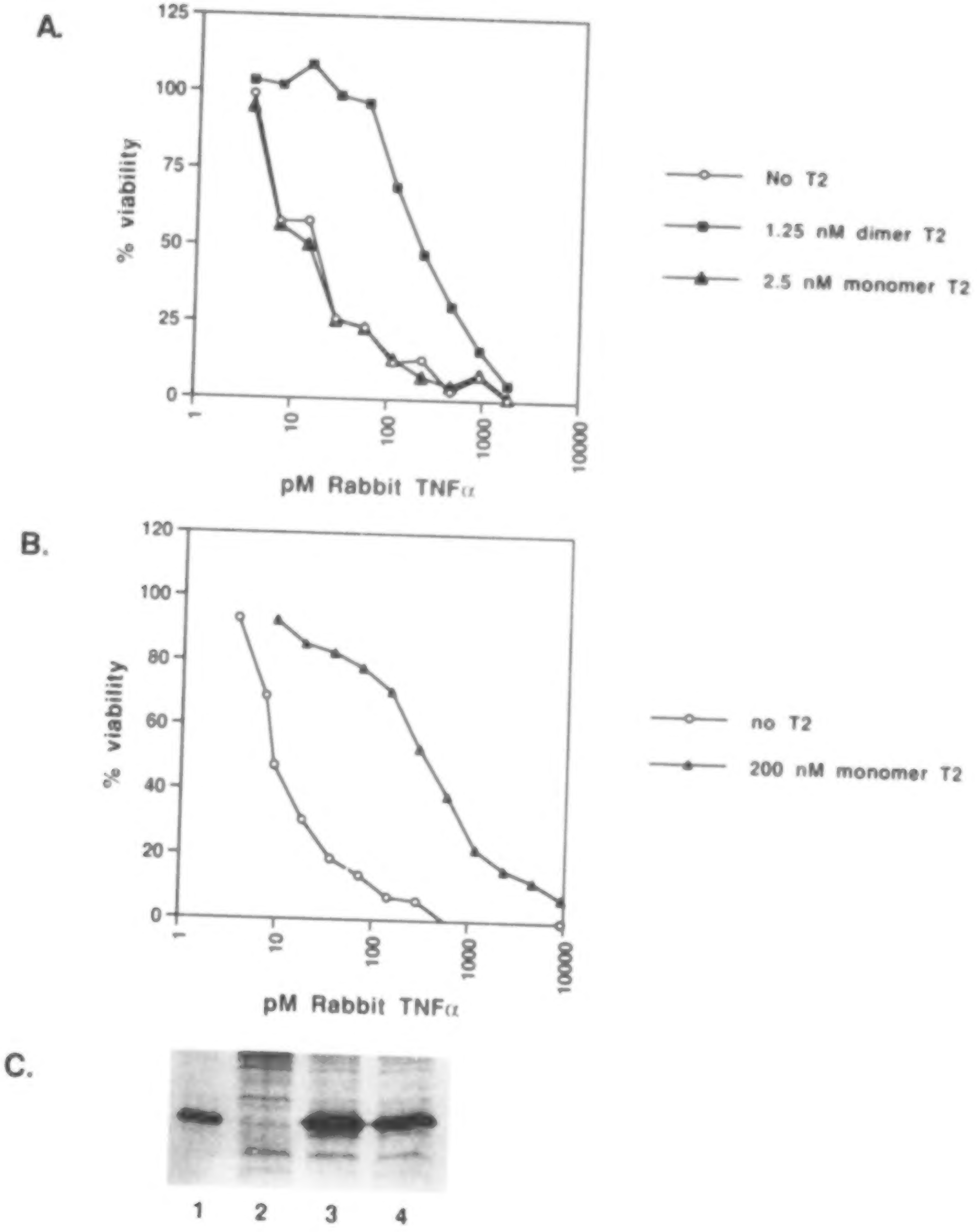

A.
L929-8
% Increase in viability
100
80
60
40
20
0
10
100
1000
10000
100000
M-T2:MuTNF
B.
L929-8
% viability
100
75
50
25
0
0.1
1
10
100
1000
M-T2:RaTNF
C.
RK-13
% viability
100
75
50
25
0
0.1
1
10
100
M-T2:RaTNF

Figure IV-10. Protection Against TNF-α Induced Cytolysis by Monomeric and Dimeric M-T2 Protein

A. Actinomycin-D sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF alone (circles) or in the presence of 5 nM dimeric M-T2 (squares) or 10 nM monomeric M-T2 (triangles), and cell viability determined by neutral red uptake as outlined under "Materials and Methods". Each point represents the mean of quadruplicate determinations.

B. Actinomycin-D sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF alone (circles) or in the presence of 200 nM monomeric M-T2 (triangles).

C. ^{35}S-labeled supernatants from VV-T2RaTNF-infected cells were incubated with no MT2 (lanes 1,2), or 25 ng dimeric M-T2 (lane 3) or monomeric M-T2 (lane 4), immunoprecipitated with rabbit anti-MT2 antisera (lanes 2-4) or anti-rabbit TNF antibody (lane 1), analyzed by SDS-PAGE as outlined under "Materials and Methods". The autoradiogram showing immunoprecipitated rabbit TNF is displayed.


A.
125
100
75
50
25
0
% viability
1
10
100
1000
10000
pM Rabbit TNFα
No T2
1.25 nM dimer T2
2.5 nM monomer T2
B.
120
100
80
60
40
20
0
% viability
1
10
100
1000
10000
pM Rabbit TNFα
no T2
200 nM monomer T2
C.
1
2
3
4

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CHAPTER V - MUTATIONAL ANALYSIS OF THE LIGAND-BINDING DOMAIN OF M-T2 PROTEIN

INTRODUCTION

The TNFR homolog of myxoma virus, M-T2 has been shown to be an important virulence factor for myxoma virus by virtue of the fact that the majority of rabbits infected with a recombinant myxoma virus in which both copies of the M-T2 gene were inactivated by insertional mutagenesis, were able to mount a more effective immune response and completely recover from infection (1). In the preceding chapters, secreted M-T2 protein has been shown to be species specific and to inhibit the cytolytic activity of rabbit TNF, but not the mouse or human ligands. Moreover, M-T2 is secreted from infected cells as both a monomer and dimer, each of which binds rabbit TNF with an affinity comparable to that of TNF with its cellular receptors; however, the dimeric form is a much more effective inhibitor of TNF activity, presumably because it is able to block oligomerization of membrane receptors by the trimeric TNF ligand. Since it was difficult to obtain enough secreted M-T2 protein from myxoma-infected cells for biochemical analysis, full length M-T2 and various deletion mutants were expressed from recombinant vaccinia viral vectors which do not express an endogenous TNFR-homolog (2, 3). Here we use mutational analysis of the M-T2 protein to map the minimal ligand binding domains of M-T2 to the first three of the four CRDs at the N-terminus of the protein and show that the C-terminal 24 amino acids are unexpectedly required for proper secretion of M-T2 as an N-linked glycoprotein.

RESULTS

M-T2 Protein is N-Glycosylated

Although the predicted molecular mass, based on amino acid composition, of unmodified, secreted M-T2 protein is 33.4 kDa, M-T2 secreted from infected cells migrates as a diffuse 55-65 kDa band on reducing SDS polyacrylamide gels. Moreover, four potential N-glycosylation sites are predicted from its sequence, suggesting that M-T2 is a glycoprotein. The glycosylation of M-T2 protein was studied to resolve the discrepancy between observed and predicted molecular weights, and to characterize further M-T2 secretion. Myxoma T2 protein purified from supernatants of VV-MT2 infected cells was treated with the enzyme N-glycosidase F and examined by immunoblotting. Figure V-1A shows a Western blot of purified M-T2 treated and untreated with N-glycosidase F, and probed with anti-MT2 antisera. N-glycosidase F cleaves asparagine linked N-glycans (4), leaving an aspartic acid residue at the site of N-glycosylation in the polypeptide backbone. Untreated M-T2 protein migrated as a 55-65 kDa diffuse band, whereas the N-glycosidase F digested protein collapsed into a 51 kDa distinct band, indicating that native secreted M-T2 protein is indeed N-glycosylated. The difference in signal between N-glycosidase F digested and undigested M-T2 protein might relect variability in the ability of the antibody to recognize the glycosylated and deglycosylated protein, or alternatively, proteolysis of M-T2 could have occured by contaminants within the enzyme preparation during deglycosylation. The latter hypothesis is unlikely, though, as both M-T2 species gave similar signals when analyzed by silver staining of SDS polyacrylamide gels. Although the mobility observed by reducing SDS-PAGE suggests the observed mass of M-T2 to be 55-65 kDa, when native M-T2 protein was analyzed by the more definitive ultracentrifugation sedimentation and mass spectroscometry techniques, the observed mass of native, monomeric M-T2 was calculated to be approximately 40 kDa. Since the difference in mobility between undigested and N-glycosidase F digested protein was close to 9 kb, the extent of N-glycosylation alone can completely account for the discrepancy between the calculated and observed molecular mass of M-T2 protein.

Although M-T2 protein does not contain proline rich regions typical of O-linked glycosylation sites, 20% of the amino acid residues are serine or threonine which could be potential sites for O-glycosylation. To determine whether the 51 kDa N-glycosidase F-digested T2 protein might be O-glycosylated as well, M-T2 blots were probed with biotin labeled lectins. Concavalin A (ConA) recognizes mannose residues added cotranslationally in the endoplasmic reticulum and is specific to N-linked glyoproteins (5), whereas *Ricinus communis* agglutinin (RCA) detects galactose residues and is a marker for both N- and O-linked glycoproteins. When nitrocellulose blots of N-glycosidase F digested and undigested M-T2 protein were probed with the two lectins, both the biotin-labeled Con A and RCA bound the glycosylated control IgG2a,κ, heavy and light chains, and myxoma T2 protein as expected, confirming the M-T2 N-glycosylation (Fig. V-1, B and C). After digestion with N-glycosidase F, binding of the biotin-labeled conA was reduced to background levels (Fig. V-1B, lane 2) indicating that N-glycosidase F digestion had removed all the N-linked sugar residues. In addition to binding untreated M-T2 protein, some biotinylated RCA also bound the N-glycosidase F digested M-T2 protein, although at considerably reduced levels compared to undigested M-T2 (compare lanes 2 and 3 of Fig. V-1C), indicating that although there may be some minor level of O-linked sugar residues, O-glycosylation does not contribute significantly to the sugar component.

The purified M-T2 protein was also digested with the enzyme endoglycosidase H (Endo H). For N-linked glycoproteins, oligomannose structures are added co-translationally onto the polypeptide chains within the endoplasmic reticulum, and the resulting glycoproteins are either secreted from cells with oligomannose sugar structures, or are further processed within the Golgi apparatus and secreted with complex or hybrid oligosaccharide chains (6). Endo H hydrolyzes most oligomannose and hybrid-type sugar chains on N-linked glycoproteins, but not complex type chains (4). M-T2 protein, purified from supernatants from VV-MT2 infected was digested with Endo H. Surprisingly, instead of one Endo-H resistant or sensitive species, four distinct Endo-H sensitive species of M-T2 protein were observed, migrating between 58 and 51 kDa. Since there are four potential N-glycosylation sites predicted from M-T2 amino acid sequence, this data suggests that all four glycosylation sites are used; however, during trafficking through the Golgi network, they may only be partially processed to Endo H resistant complex oligosaccharide chains. M-T2 protein thus appears to be secreted as a mixture of glycoforms, with both Endo H resistant complex and Endo H sensitive high-mannose and hybrid sugar chains. Processing may be random at the different sites, and processing to complex Endo-H resistant forms appears to occur at multiple N-glycosylation sites.

We next tested whether these multiple Endo-H sensitive forms were a consequence of expressing M-T2 protein from a vaccinia virus construct as opposed to the native myxoma virus. Figure V-2A shows [^{35}S]-met/cys M-T2 protein immunoprecipitated from supernatants from myxoma- (vMyxlac)-infected BGMK cells and treated or untreated with Endo H. No reactive protein was observed immunoprecipitated from mock-infected cells, or cells infected with the T2-inactivated virus, vMyxt2g, as expected (lanes 1-4). However, secreted M-T2 protein was readily detected in supernatants harvested from vMyxlac-infected cells radiolabelled 0-4 hours (lane 5) and 4-8 hours (lane 7) post infection. Moreover, upon digestion with Endo-H, the four distinct M-T2 glycoforms migrating between 51-59 kDa were observed (lanes 6, 8, respectively). More M-T2 protein was observed secreted during the first four hours post infection, compared to the 4-8 hour time interval, consistent with the expression of a stable M-T2 mRNA from an early viral promoter. Furthermore, the Endo H-digestion profile of secreted M-T2 protein was identical between myxoma- and VV-MT2-infected cells (Fig. V-1D, and Fig. V-2A lanes 6 and 8), suggesting that poxvirus infection might in general attenuate the extent of terminal sugar modifications of M-T2.

The various enzymes responsible for oligosaccharide processing are present at specific locations within the endoplasmic reticulum and Golgi complex (4, 7). Within the Golgi network N-linked glycoproteins from the endoplasmic reticulum are remodelled to complex and hybrid forms, high mannose structures are processed by α-mannosidase II to complex Endo H resistant forms, and O-linked sugars are added. Therefore, we tested to see whether intracellular M-T2 protein exhibited the multiple distinct Endo-H sensitive M-T2 glycoforms observed in the secreted protein. Figure V-2B shows that when M-T2 protein is immunoprecipitated from cell lysates, only Endo H sensitive M-T2 protein was observed in the intracellular fraction from vMyxlac- (lanes 1,2) or VV-MT2-infected cells (lanes 5-6), suggesting that processing of M-T2 protein to the various glycoforms is a late event and the different glycoforms do not accumulate within the cell.

Construction of Vaccinia Viruses Expressing C-Terminal Deletions in M-T2 Protein

To further characterize M-T2 and ascertain which region of the protein is critical for binding and inhibition of rabbit TNF, a series of recombinant vaccinia viruses were constructed to express various staggered C-terminal deletions in M-T2 protein. M-T2 amino acid homology with other members of the TNFR superfamily is limited to the N-terminal region which contains the four cysteine rich domains (CRDs) characteristic of the motifs present in the ligand binding domains of other members of the TNFR superfamily (1). The C-terminal 132 amino acid sequence of M-T2 is unique, although it is relatively conserved in other intact poxvirus homologs of M-T2. The strategy used to generate these mutants is outlined in Figure V-3A and is described in the Materials and Methods. The structure of VV-MT2, showing the N-terminal leader cleavage site, the four predicted N-glycosylation sites, and the four CRDs, plus the structure of the control virus, VV-601, with only a lacZ cassette inserted into the *tk* locus of vaccinia virus are outlined in

Figure V-3B and the vaccinia viruses expressing the various C-terminal truncations from a common synthetic late promoter are shown in Figure V-3C. Vertical lines indicate the location of cysteine residues present in M-T2 parental sequence. The viruses are named according to the first amino acid codon deleted within the truncated protein, and deletions extend up to the removal of all but the first 112 amino acids (VV-MT2-ΔL113) to the loss of only the C-terminal 24 amino acids (VV-MT2-ΔD303). The protein expressed from VV-MT2-ΔL113 contains the first two CRDs, plus eight amino acids (including two cysteines) of the third repeat. VV-MT2-ΔN169 and VV-MT2-ΔE174 have deletions beginning within the fourth CRD and include 22 amino acids and 27 amino acids (each with 2 cysteines) of the fourth repeat, respectively, whereas the remainder of the truncations contain deletions spanning the unique 132 amino acid C-terminal domain of M-T2.

Supernatants from cells infected with the different vaccinia viral constructs were tested for the presence of truncated M-T2 protein. Although M-T2 protein is the major secreted protein from cells infected with VV-MT2, the amount of truncated protein secreted from cells infected with the viruses expressing all of the M-T2 C-terminal deletions was consistently reduced at least ten-fold. Figure V-4A shows a Western blot of supernatants from cells infected with vaccinia viruses expressing the different M-T2 truncations. Secreted proteins were precipitated with methanol:acetone, resolved by SDS-PAGE and probed with affinity purified rabbit anti-MT2 antibody. Lane 1 shows that the antibody consistently cross-reacts with a 70-72 kDa species present in control VV-601 supernatants, as well as supernatants from cells infected with all the VV-MT2 variants. We believe this unknown protein to be either derived from the newborn calf serum used to culture BGMK cells or else is a cross-reactive cellular protein secreted from these cells. In order to compare the mobility of secreted M-T2 to the various deletion constructs, it was necessary to load 10X more protein to detect the secreted variants. Thus, lanes 2-10 show M-T2 variant proteins secreted from the equivalent of 5 x 10^4 VV-MT2 infected cells (lane 2) and 5 x 10^5 VV-MT2-ΔC-terminus-infected cells (lanes 3-10). The blot was over-exposed to bring out any minor bands present in some of the lanes. Full length M-T2 appears as a major 55-65 kDa diffuse band (lane 2), and a minor 50 kDa variant, which may reflect underglycosylated M-T2 protein or a low level of cell lysis resulting in protein degradation. Each of the deletion mutants migrated with an apparent molecular weight larger than that predicted from the amino acid sequence, suggesting that all of these proteins were glycosylated to some extent. Moreover, the different M-T2 truncations appeared as multiple bands which may reflect either glycosylation variants of M-T2 protein or limited release via disruption of infected cells. In ΔD303 (lane 8), only the C-terminal 24 amino acids of M-T2 are deleted which still results in a reduction of protein secretion by at least an order of magnitude from infected cells. The amount of M-T2 variant protein secreted with each virus varied from infection to infection, and in some instances almost no protein was observed in infected supernatants (not shown). Table V-1 summarizes the predicted and apparent sizes of the different M-T2 truncations observed to be secreted into supernatants.

Since compared to full length M-T2 protein, significantly less of the truncated variants was observed in supernatants, cell lysates were examined to see whether the different proteins might be expressed at comparable levels but might be retained as intracellular proteins. Figure V-3B shows a Western blot of lysates from equivalent numbers of cells infected with the various recombinant vaccinia viruses. The antibody did not react with any proteins present in control VV-601 infected cells (lane 10), but did react with 59 kDa intracellular M-T2 protein. The different C-terminal truncations were all preferentially retained as intracellular proteins within infected cells (lanes 3-9); observed sizes of the intracellular mutants are also summarized in Table V-1. The intracellular M-T2 truncations, immunoprecipitated from cell lysates, were then digested with Endo H. The different variants all remained fully Endo H sensitive (not shown), confirming that they were glycosylated and most likely accumulated within the endoplasmic reticulum or possibly the cis Golgi. For all the truncations, including ΔD303 with only the C-terminal 24 amino acids deleted, significantly more intracellular protein compared to intracellular full-length M-T2 protein was consistently dectected. We suggest that a sequence residing within the C-terminal 24 amino acids is important for the efficient trafficking of M-T2 protein from infected cells to the extracellular milieu, but the precise domain of this sequence remains to be mapped.

MT2-ΔL113, the shortest deletion mutant, contains only 34% of full-length M-T2 protein sequence. The difficulty in observing ΔL113 protein as both intracellular and secreted forms may reflect a lower amount of protein or a decreased ability of the polyclonal antibody to detect the truncated protein. To enhance the ability to analyze the shortest truncations, ΔL113, ΔN169 and ΔE174 were first immunoprecipitated from cell lysates, and the immunoprecipitated proteins examined by Western analysis (Fig. V-4B lanes 11-14). In lane 12, an 11 kDa diffuse band is observed from immunoprecipitates of VVMT2-ΔL113-infected cell lysates that is absent from immunoprecipitates of control VV-601 infected cell lysates. The predicted size of ΔL113 protein is 10.1 kDa, but it does include one predicted N-linked glycosylation site. Species that migrate at 23 and 30 kDa are detected for ΔD169 and ΔE174, as expected, and the species around 65 kDa observed in all lanes represents the antibody used to immunoprecipitate the M-T2 proteins.

For intracellular ΔE174, ΔI260, ΔI279 and ΔD303, higher molecular weight species were also observed on the Western blot, most likely reflecting dimeric forms of these proteins. Although the cells were lysed in Laemmli sample buffer containing dithiothreitol, there may have been incomplete reduction of all the disulphide linkages in the intracellular proteins resulting in some residual dimers. When lysates were analyzed at an increased dithiothreitol concentration, these higher molecular weight species disappeared (data not shown). Thus, for, ΔE174, the monomeric form migrated between 22-28 kDa, whereas the dimeric species migrated at 47-56 kDa. For ΔI260, ΔI279 and

ΔD303 (lanes 6-8), 47-58 kDa and 88-96 kDa bands were observed but the size of the upper band can only be approximated because it is out of the linear range for estimating molecular weights. For ΔN169, the band observed at 43 kDa is the predicted size of the dimeric form, whereas the 18 kDa band may correspond to some unglycosylated ΔN169. Note that full length M-T2 is secreted from infected cells as a mixture of both monomer and dimer. Twenty-seven cysteines are present in full-length M-T2, most of them presumably involved in intramolecular disulphide linkages, especially in the folding of the N-terminal cysteine-rich repeats. In the different M-T2 truncations, the disulphide linkages within the C-terminus would be disrupted, as cysteines were deleted, which in turn could effect the oligomeric status of the processed M-T2 protein. When cells were lysed with Laemmli loading buffer without dithiothreitol, and examined by non-reducing SDS-PAGE, inefficient solubilization of the intracellular proteins was observed. However, multiple forms, monomers, dimers, and in some cases, trimers, of the different truncations was observed (data not shown), confirming that some of the disulphide linkages are indeed intermolecular.

Construction of Vaccinia Viruses Expressing M-T2 Protein with Deletions of the Cysteine-Rich Repeats

A series of vaccinia viruses were constructed expressing successive deletions of each of the four cysteine-rich domains, using the polymerase chain reaction to generate the internal deletions, as outlined in the Materials and Methods. In Figure V-5 the different CRD deletions are outlined, showing the predicted leader cleavage site and N-linked glycosylation sites. The secreted (Fig. V-6A) and intracellular (Fig. V-6B) proteins were analyzed following immunoprecipitation with polyclonal anti-MT2 antisera from [^{35}S]-met/cys-labelled cells infected with the recombinant vaccinia viruses expressing the four deletions. Immunoprecipitates from control VV-601-infected cells (Fig. V-6 A, B, lane 1) identify minor non-M-T2 proteins which bind the antibody or Protein A beads. Secreted, full length M-T2 migrates as an approximately 60 kDa diffuse band (Fig. V-6A, lane 2), and after 24 hours post infection, essentially all of the intracellular M-T2 protein had been secreted (Fig. V-6B, lane 2). Secreted and intracellular forms of the ΔCRD1, ΔCRD2 and ΔCRD3 proteins were observed as 60 kDa, 57 kDa and 59 kDa bands respectively (Fig. V-6 A and B lanes 3-5); however, neither secreted nor intracellular ΔCRD4 protein could be detected. Deletion of this fourth cysteine rich repeat may result in aberrant folding and subsequent degradation of this protein or possibly destabilize the truncated mRNA species. ΔCRD2 contains only three of the four potential glycosylation sites and this variant migrates more quickly than the other ΔCRD mutant proteins (Fig. V-6 A and B lane 4). Moreover, slightly more ΔCRD2 protein was detected in the supernatants compared to ΔCRD1 and ΔCRD3, and this was accompanied by a subsequent reduction of intracellular ΔCRD2 (compare lanes 4, Fig. V-6 A and B). Nevertheless, the efficiency of secretion of all of the ΔCRD proteins has been reduced compared to full length M-T2, possibly because of altered protein/protein interactions with elements of the secretory pathway. However, much more ΔCRD protein was secreted compared to any of the C-terminal truncations, and the multiple bands of lower MW that were observed with the secreted C-terminal truncations were not observed with the secreted ΔCRD mutants. This data shows that for the three ΔCRD mutants in the first three domains, recombinant protein was being secreted from virus-infected cells. Thus, we conclude that for three of the four ΔCRD mutants, M-T2 variant protein is efficiently produced and secreted from cells infected with the viral vectors, although the efficiency of protein secretion may be somewhat less than full-length MT2 from VV-MT2 infected cells.

M-T2 Protein Variants Containing C-Terminal Truncations, but not Deletions of any of the N-Terminal CRDs Bind Rabbit TNF

The M-T2 protein variants, thus created, were tested for their ability to bind and co-immunoprecipitate with rabbit TNF. The vaccinia virus construct VV-T2RaTNF was used as a source of rabbit TNF as it directs the efficient production and secretion of mature rabbit TNF protein from infected cells. BGMK cells were infected with VV-T2RaTNF in the presence of $[^{35}S]$-met/cys, and the supernatants containing $[^{35}S]$-labelled rabbit TNF were harvested. These supernatants were then combined with unlabelled samples prepared from cells infected with the various M-T2 deletion constructs, and then proteins were immunoprecipitated with affinity purified anti-MT2 antibody, thus assaying the ability of the M-T2 variants to bind and co-precipitate with rabbit TNF. In Figure V-7A, the ability of secreted full length M-T2 protein, or the different M-T2 variants, to bind and co-precipitate rabbit TNF is shown. Supernatants from VV-MT2, VV-MT2ΔC-terminus or VV-MT2ΔCRD infected cells were tested for their ability to co-precipitate rabbit TNF as outlined in the Materials and Methods. In lane 1, radiolabelled rabbit TNF was immunoprecipitated with polyclonal anti-rabbit TNF antibody, and migrated as an 18 kDa band by SDS-PAGE, serving as a marker for labelled rabbit TNF protein as shown. In lane 2, $[^{35}S]$-labelled rabbit TNF was combined with proteins secreted from cells infected with virus, VV-601, and no rabbit TNF could be detected co-precipitating with the anti-MT2 antibody. However, rabbit TNF was able to bind and co-precipitate with secreted full length M-T2 protein (lane 3) and ΔE174, ΔY236, ΔI260 and ΔD303-M-T2 variants (lanes 5-8) with comparable efficiency. In stark constrast, however, the ΔL113 variant was unable to co-precipitate any rabbit TNF (lane 4). As a confirmation of this result, ΔL113 was combined with purified rabbit TNF, but anti-rabbit TNF antibody also did not co-immunoprecipitate ΔL113, under conditions in which native MT2 protein was co-immunoprecipitated (data not shown). Since ΔL113 is the first mutant to extend into the third CRD, we conclude that excision of C-terminal sequences up to and including deletion of sequence within the fourth CRD (i.e. ΔN169 and ΔE174) does not alter the ability of M-T2 to bind rabbit TNF. This is in direct contrast to removal of any of the first three CRDs (Fig. V-7A, lanes 9-11). The homologous cysteine rich repeats comprise the ligand binding domain of the cellular TNFRs, and removal of either CRD1, CRD2 or CRD3 resulted in an M-T2 variant incapable of binding and co-precipitating rabbit TNF.

Because the different M-T2ΔC-terminus mutants were preferentially retained as intracellular proteins, we tested to see whether the intracellular variants were able to co-precipitate with rabbit TNF. The results of an experiment in which cellular protein lysates were mixed with [^{35}S]-labelled rabbit TNF are shown in Figure V-7B. Lane 1 shows 18 kDa rabbit TNF immunoprecipitated with polyclonal anti-rabbit TNF antibody. When [^{35}S]-met/cys-labelled supernatants from VV-T2RaTNF-infected were tested alone, or combined with lysates from mock- or VV-601-infected cells and immunoprecipitated with anti-MT2 antibody, rabbit TNF was not co-precipitated (lanes 2,3 and 5). However, rabbit TNF did co-precipitate with M-T2 antibody when combined with lysates from VV-MT2-ΔN169, ΔE174, ΔY236, ΔI260, ΔI279 and ΔD303-infected cells (lanes 7-12), but not when combined with lysates from VV-MT2-ΔL113-infected cells (lane 6). Note that when 24 hour lysates of VV-MT2-infected cells were used to co-precipitate rabbit TNF, only a faint band corresponding to rabbit TNF was observed (lane 4), because by this time almost all of the wild-type M-T2 protein had been effectively secreted into the medium. Thus, for all of the C-terminal truncations, from ΔN169 to ΔD303, both secreted and intracellular proteins were able to bind and co-precipitate rabbit TNF, but the ΔL113 variant from either fraction did not detectably interact with rabbit TNF.

MT2 Protein Truncation Variants that Bind TNF, but not Deletions of the Internal Conserved Repeats, also Inhibit the Cytolytic Activity of Rabbit TNF

The various MT2 deletions were tested for their ability to inhibit the cytolytic activity of rabbit TNF. Actinomycin-D sensitized L929-8 cells, which are sensitive to the cytotoxic effects of rabbit TNF, were co-incubated with increasing concentrations of rabbit TNF and 500 ng/ml M-T2 deletion protein for ΔN169 to ΔD303 and 100 ng/ml for ΔD113, present in supernatants, or 500 ng/ml purified M-T2 protein and cell viability was assayed after 18 hours. As shown in Figure V-8A, rabbit TNF (filled circles), at concentrations of 10 pM and higher was completely cytolytic to L929-8 cells. The ΔL113 protein (filled triangles) was completely unable to protect against TNF-induced cytolysis, while the M-T2 C-terminal truncations ΔE174, ΔY236, ΔI260 and ΔD303 were able to protect L929-8 cells from lysis by rabbit TNF, with protection profiles similar to purified, full-length M-T2 protein. Thus, all the M-T2 variants, except ΔL113, provided full protection against the cytotoxic effects of 100 pM rabbit TNF. Previously, we found that full-length M-T2 protein is secreted from infected cells in roughly equal proportions as both a monomer and a dimer, but only the dimeric form is an effective inhibitor of TNF signalling, probably because it can more effectively prevent dimerization of cellular receptors. SDS-PAGE analysis of the M-T2 truncations under non-reducing conditions suggested that these proteins were secreted as dimers and higher oligomers (data not shown). The mutant ΔN169, truncated within the fourth conserved repeat but still containing the first two cysteines of this repeat, was able to protect L929-8 cells from lysis (filled circles), although not as effectively as the longer M-T2 variants. Since the detailed oligomeric status for the different M-T2 truncations are unknown, we cannot deduce if this reduced ability to protect might be due to altered ratios of monomers to oligomers. However, we can conclude that removal of the C-terminus of M-T2, up to the middle of the fourth conserved repeat, does not alter the ability to bind and inhibit rabbit TNF.

In contrast to the C-terminal deletions, removal of any of the first three CRDs resulted in the complete ablation of the ability to protect L929-8 cells from lysis by rabbit TNF. In Figure V-8B, no difference was detected between cell lysis with increasing amounts of TNF alone or in the presence of 500 ng/ml ΔCRD1, ΔCRD2 or ΔCRD3 protein. This result was consistent with the inability of any of these deletion mutants to co-precipitate rabbit TNF. Thus, we conclude that the ability of M-T2 protein to inhibit rabbit TNF is dependent solely upon the N-terminal cysteine rich repeats, and the first three CRDs are absolutely essential for this inhibitory activity.

DISCUSSION

M-T2 protein, secreted from cells infected with either myxoma or recombinant MT-2 expressing vaccinia constructs, is a soluble N-glycosylated inhibitor of rabbit TNF. The secreted protein was only partially sensitive to Endo H, indicating it was exported as a mixture of both complex and hybrid or high mannose oligosaccharide structures. Intracellular, full length M-T2 protein was fully Endo H sensitive indicating that the Endo-H resistant fraction was secreted quickly after terminal processing. Although it has not been rigorously shown that in poxvirus-infected cells all processing to Endo H resistance occurs only within the Golgi network, it is reasonable to suggest that most of the intracellular M-T2 protein is located at a pre-Golgi site, presumably as a pool awaiting extracellular transport within the endoplasmic reticulum. Moreover, all the M-T2 C-terminal truncations studied here, unlike the full length M-T2 protein, were effectively retained as intracellular glycoproteins which were fully Endo H sensitive, suggesting they had not yet entered the Golgi network. Thus, we suggest that the C-terminal 132 amino acids of M-T2, which is not homologous to any cellular receptor, functions in part to direct the trafficking of M-T2 to the extracellular medium. Alternatively, the C-terminal truncations might be correctly folded at their N-termini because of the disulphide linkages within the CRDs, but incorrectly folded at their C-termini. Calnexin is a chaperone which retains misfolded or incorrectly assembled proteins within the endoplasmic reticulum (8-12). Misfolding only at the C-termini could potentially have resulted in the M-T2 mutants being selectively retained within the endoplasmic reticulum by calnexin or another chaperone, yet still capable of binding rabbit TNF. If this were the case, one would not expect to see accumulation of the truncations within the cell as misfolded proteins are often targeted for degradation. However, each of the M-T2 truncations accumulated within the cell. It would be interesting to test if the intracellular retention of the M-T2 truncations was due to its association with calnexin.

Like M-T2 protein, the p55 and p75 TNFRs possess predicted sites for N-linked glycosylation, although very little is known about the role of TNFR glycosylation. The human p55 TNFR possesses three potential sites for N-linked glycosylation (13, 14), whereas the p75 TNFR has potential O-linked glycosylation sites in addition to three N-linked sites (15). Although mouse TNF is naturally glycosylated, human TNF is not; both human and mouse LTα are glycosylated (16). Glycosylation does not appear to be essential for biological function in that recombinant mouse TNF and human LTα produced in bacterial expression systems show full cytolytic activities (17, 18). However, only recently has the significance of protein glycosylation begun to be understood, and in addition to affecting the structure of the protein and resistance to extracellular protease digestion, oligosaccharides have been found to modify ligand-receptor interactions and in some instances be directly involved in high affinity binding (6, 19, 20). Recently, different glycoforms have been identified for the human, soluble p75 TNFR, one form was only N-glycosylated whereas the other was both N- and O-glycosylated (21). Moreover, an O-linked glycosylated species of TNF has also been identified (22). The functional significance of these TNF and TNFR glycoforms remains to be determined.

Various attempts were made to determine whether N-glycosylation was important for M-T2 interaction with rabbit TNF. Unfortunately, treatment of infected cells with tunicamycin at concentrations which inhibited N-glycosylation, also inhibited the production and secretion of viral proteins from infected cells (not shown). However, the crystal structure for the related p55TNFR and TNFβ has been solved (23), and sugar residues are not believed to be critical for ligand binding *per se*. It is unknown if sugar residues are directly involved in M-T2 binding TNF, but it should be noted that oligosaccharide chains may modify the interactions of other ligands with their receptors or binding proteins (6, 19).

The various M-T2 truncation and deletion mutants were tested to determine which domains of M-T2 were essential for ligand binding and inhibition of cytolysis. The M-T2 C-terminal deletions up to and including truncations within the middle of the fourth CRD retained the ability to bind rabbit TNF and inhibit its biological activity. Elsewhere, we have shown that full length M-T2 protein is secreted from infected cells as both a monomer and a disulphide-linked dimer; the dimer, however, is by far the more effective TNF-inhibitor, presumably because the M-T2 dimer/TNF trimer complex cannot induce oligomerization of membrane TNF receptors. Thus, truncations within the C-terminus would be expected to disrupt not only protein domains but disulphide bridging as cysteines were removed, and various oligomeric forms, including dimers and in some instances trimers, of the M-T2 deletion mutants could be detected in cell lysates (Fig. V-4B), suggesting that the CRD sequences mapped in this study represent true ligand-interaction domains. The different C-terminal truncations, ΔD303 to ΔE174 all inhibited the cytolytic activity of rabbit TNF on L929-8 cells at levels comparable with full length M-T2 protein. The decreased ability of ΔN169 to inhibit TNF, despite its ability to co-immunoprecipitate TNF, might reflect variations in oligomeric status or alternatively, steric hindrance might occlude the efficient binding of two TNF monomers per trimer.

The TNF binding domain of M-T2 protein is, as predicted, confined to the N-terminus within the CRDs that exhibit homology with the CRDs of the ligand binding domains of other members of the TNFR superfamily. C-terminal truncations up to and within the fourth CRD of M-T2 did not effect the ability of M-T2 to bind or inhibit TNF. However, deletion of either the first, second or third CRD completely abolished the ability of M-T2 protein to co-immunoprecipitate rabbit TNF or inhibit its cytolytic activity, suggesting that each of the first three CRDs of M-T2 protein are essential for TNF binding. Often, incorrectly folded proteins within cells are targeted for degradation, yet the M-T2 variants with deletions of either the first, second or third CRD were efficiently expressed and secreted from infected cells. In contrast, when cells were infected with a vaccinia virus engineered to express M-T2 protein with the fourth CRD deleted, no intracellular or secreted M-T2 protein could be detected. In this case, deletion of this domain may have caused a structural change resulting in an incorrectly folded protein degraded soon after synthesis. Alternatively, mRNA stability sites may be located within the coding sequence of the N-proximal region of the fourth CRD, and the absence of any expressed ΔCRD4 protein could thereby be due to reduced levels of M-T2 message. C-terminal truncations ΔN169 and ΔE174, containing only 47% and 57% of the fourth CRD respectively, were efficiently expressed and each of these variants did bind and inhibit rabbit TNF. Thus, sequences within the first three CRDs of M-T2 protein are clearly important for TNF binding and inhibition, but a complete fourth CRD domain is apparently not essential.

Similarily, the first three CRDs appear to be essential for the p55 TNFR binding to TNF and LTα, whereas the fourth CRD is not essential, and its removal has been reported to have no effect on ligand binding, or decrease it only ten-fold (24, 25). Mutant p55 TNFRs containing deletions of either the first, second or third CRDs did not bind TNF or LTα, whereas the TNFR derivative in which the fourth CRD was deleted bound both ligands and inhibited the cytolytic activity of TNF on WEHI 164 cells (24). Moreover, Corcoran *et al* (24) report that the removal of the fourth CRD did not alter the affinity of the p55 TNFR for TNF when assessed by Scatchard analysis. In a similar experiment, when fusion proteins of the p55 ligand binding domain, or variants containing deletions of the the first or fourth CRD, with an IgG heavy chain, were tested, it was found that deletion of the first CRD resulted in a complete loss of ligand binding, whereas deletion of the fourth CRD resulted in a 10-fold decrease in affinity for both TNF and LTα (25). Immunofusions containing only the second CRD or the second and third CRDs of the p55 TNFR were unable to bind TNF, whereas immunofusions containing all four CRDs bound and inhibited its activity (26). The importance of the first three CRDs of both the p55 and p75 TNFRs for TNF binding is reinforced in that the first two or the last two CRDs could not be replaced with the equivalent CRDs from the related nerve growth factor receptor (27). In contrast, for the related nerve growth factor receptor, it is the third and fourth CRDs which appear to play the greater role in ligand binding, in that linker insertions within these domains had the greatest effect on the receptors ability to bind nerve growth factor (28).

The crystal structure of the extracellular domain of the p55 TNFR with TNFβ provides a model for the interaction between TNF ligands and their cognate TNFRs or binding proteins (23). The extracellular domain of the receptor itself forms a very elongated end-to-end assembly of four similar folding domains, defined by the cysteine rich repeats, and contact sites with the TNF-β ligand are mainly confined to the second CRD, and to a lesser extent the third and first CRDs. The crystal structure thus confirms the deletion mutational data on the p55 TNFR, suggesting that the first three CRDS were essential for TNF binding. Moreover, in comparing M-T2 sequence with that of the p55 TNFR (1), there is good conservation of the domain boundaries and the first three CRDs can be aligned without major insertions or deletions, indicating that the structure of the p55 TNFR can provide a good model for the structure of M-T2/TNF-interactions. Thus, like the p55 TNFR, the majority of the contact sites of TNF with M-T2 would be predicted to be located within the first three domains.

In interpretating data wherein the various CRDs of the TNFRs or M-T2 protein were deleted, it cannot be ruled out that the loss in the ability to bind TNF is due to a loss of correct domain folding after deletion of an adjacent domain. Although we were unable to address this question with respect to M-T2, several groups tested various p55 TNFR deletions with conformation-dependent anti-TNFR monoclonal antibodies (24, 25), which suggested that the CRD-deleted proteins had folded correctly enough to be recognized by the epitope-specific antibodies. Moreover, the crystal structure of the p55

TNFR revealed the four CRDs as distinct entities, with little overlap or interaction between them. The six cysteines present in each repeat form three intradomain disulphide bridges evenly spaced over the length of the repeat, conferring a high degree of rigidity. Thus, the removal of one repeat would not be expected to significantly alter the conformation of adjacent repeats.

The different M-T2 mutations have been useful in mapping the domains within M-T2 which are important for TNF binding and protein secretion. In addition to inhibiting the cytolytic activities of rabbit TNF, M-T2 has also been found to have anti-apoptotic properties in virus-infected lymphocytes. RL-5 cells, a $CD4^+$ rabbit T cell line, were found to undergo apoptosis when infected with the T2-inactivated myxoma virus, vMyxt2g, but not the parental vMyxlac, expressing full length M-T2 (29). Myxoma viruses expressing the various M-T2 truncations will be useful in defining whether the anti-apoptotic activities of M-T2 overlap with the anti-TNF domains defined by deletion analysis.

Figure V-1. Glycosylation of Secreted M-T2 Protein

Purified M-T2 protein was untreated or treated with N-glycosidase F (panels A-C) or Endo H (panel D), resolved by SDS-PAGE and transferred to nitrocellulose as described under Materials and Methods. Panel A, undigested M-T2 (lane 1), N-glycosidase F digested M-T2 (lane 2) probed with anti-MT2 antibody, arrows designate undigested and digested M-T2 protein; Panels B and C, mouse IgG2a, κ (lane 1), N-glycosidase F digested M-T2 (lane 2), undigested M-T2 (lane 3), probed with con A (panel B) and RCA (panel C); Panel D, undigested M-T2 (lane 1), N-glycosidase F digested M-T2 (lane 2), probed with anti-MT2 antibody. Size standards of 112, 84, 53.2, 34.9, 28.7 kDa (Panels A-C) and 101, 83, 50.6, 35.5 kDa (Panel D) are shown.

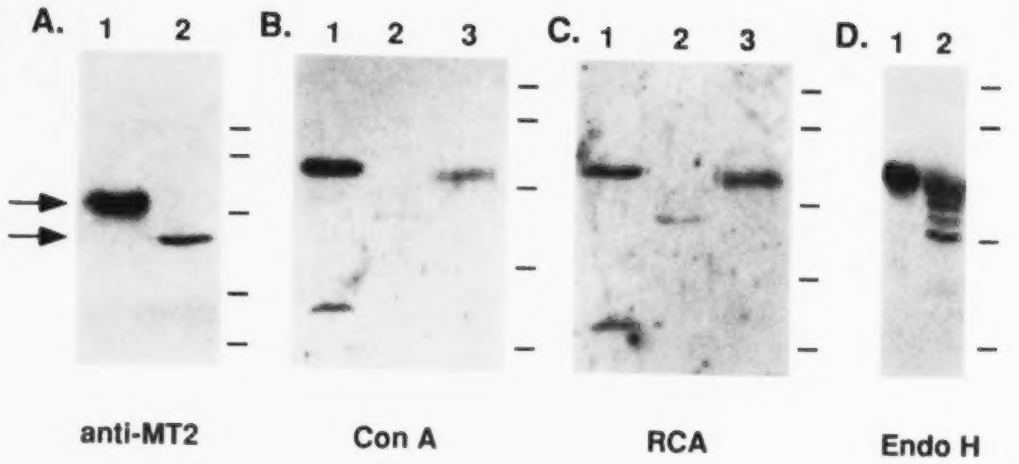

A.
1
2
anti-MT2
B.
1
2
3
Con A
C.
1
2
3
RCA
D.
1
2
Endo H

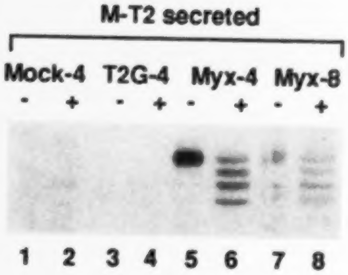
Figure V-2. Endo H Sensitivity of Secreted and Intracellular M-T2 Protein

[^{35}S]met/cys-labelled M-T2 was immunoprecipitated from supernatants (A) or cell lysates (B) of infected cells, and untreated or treated with Endo H.

A. Immunoprecipitations from supernatants from BGMK cells infected with mock (lanes 1,2), vMyxt2g (lanes 3,4), and vMyxlac (lanes 5,6) labelled from 0-4 hours post-infection, or from supernatants from vMyxlac (lanes 7,8) labelled from 4-8 hours post-infection, undigested (lanes 1,3,5,7) or digested with Endo H (lanes 2,4,6,8).

B. Immunoprecipitations from lysates from BGMK cells infected with vMyxlac (lanes 1,2), VV-601 (lanes 3, 4) or VV-MT2 (lanes 5,6) labelled from 4-8 hours post-infection, undigested (lanes 1,3,5,) or digested with Endo H (lanes 2,4,6).

A.


M-T2 secreted
Mock-4
T2G-4
Myx-4
Myx-8
- + - + - + - +
1 2 3 4 5 6 7 8

B.

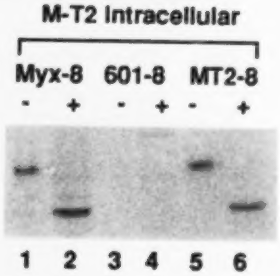

M-T2 Intracellular
Myx-8
601-8
MT2-8
- + - + - +
1 2 3 4 5 6

Figure V-3. Construction of M-T2 C-Terminal Deletions

A. Diagrammatic representation of the construction of M-T2 C-terminal unidirectional deletions, as described under Materials and Methods, showing the M-T2 gene (rectangular box) and multiple cloning site within pMTN-6; H, Hind III; S, Sal I; P, Pst I; Xh, Xho I; Xb, Xba I; B, Bam HI.

B. Structure of vaccinia (WR), showing the thymidine kinase (*tk*) locus; VV-601, with lacZ under the control of the p7.5 promoter; VV-MT2, showing M-T2 expression under the control of a synthetic late promoter (SLP) with ovals representing the four CRDs; and VV-T2RaTNF, in which the endogenous secretory leader sequence was replaced with that of M-T2 (black box) to improve the efficiency of TNF secretion from infected cells. Asterisks denote predicted N-glycosylation sites, black boxes indicate leader sequences and arrowheads designate leader cleavage sites.

C. Structure of the different M-T2 C-terminal truncations, aligned with M-T2 sequence, with vertical lines indicating the location of cysteine residues, asterisks showing the location of predicted N-glycosylation sites, a black box and arrowhead designating the M-T2 N-terminal leader sequence and leader cleavage site respectively, and rectangular boxes terminating at the point of each truncation.

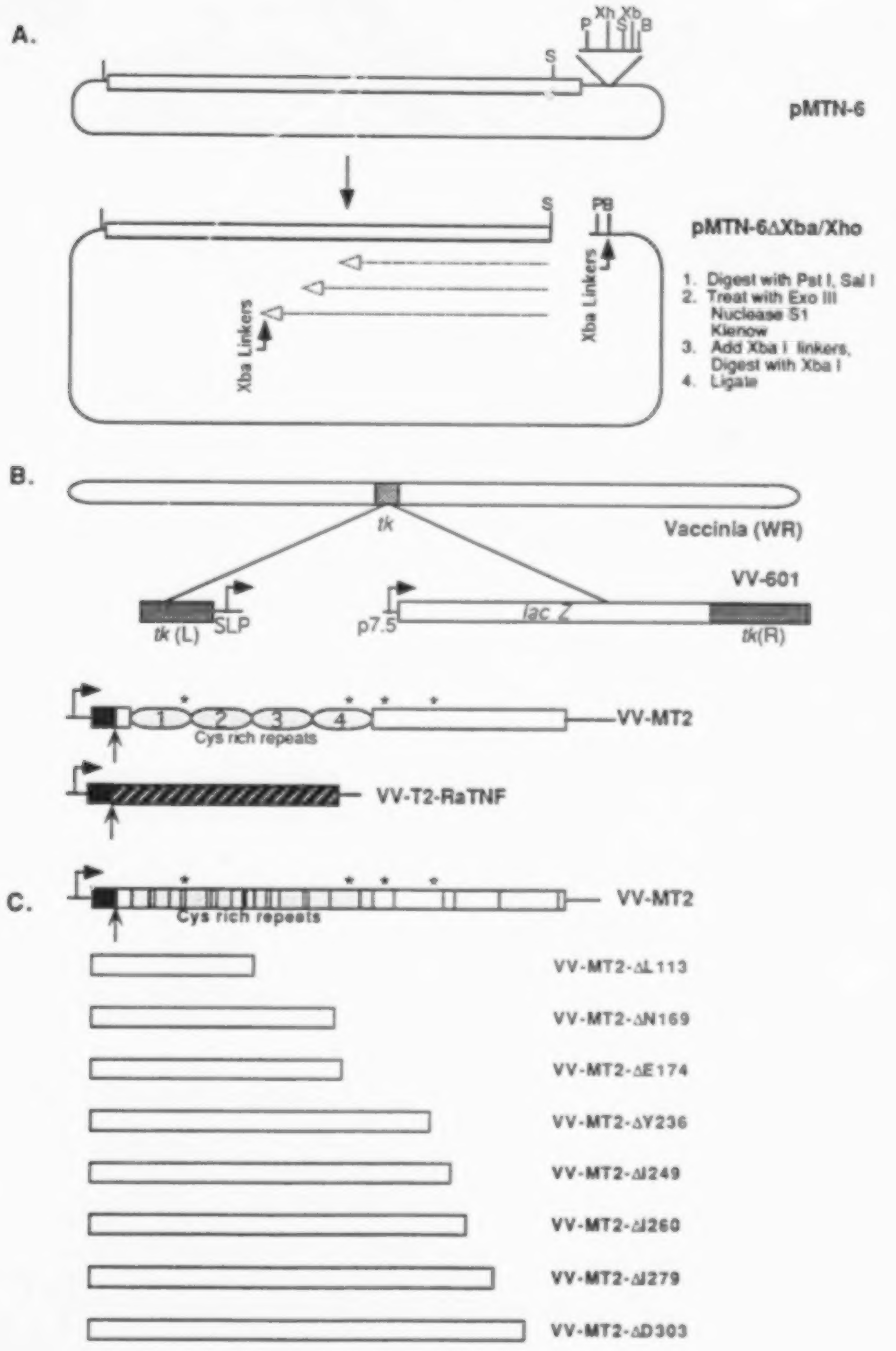

A.
P
Xh
Xb
S
B
S
pMTN-6
S
PB
pMTN-6ΔXba/Xho
Xba Linkers
Xba Linkers
1. Digest with Pst I, Sal I
2. Treat with Exo III
Nuclease S1
Klenow
3. Add Xba I linkers,
Digest with Xba I
4. Ligate
B.
tk
Vaccinia (WR)
VV-601
tk (L)
SLP
p7.5
lac Z
tk(R)
1
2
3
4
Cys rich repeats
VV-MT2
VV-T2-RaTNF
C.
Cys rich repeats
VV-MT2
VV-MT2-ΔL113
VV-MT2-ΔN169
VV-MT2-ΔE174
VV-MT2-ΔY236
VV-MT2-ΔI249
VV-MT2-ΔI260
VV-MT2-ΔI279
VV-MT2-ΔD303

Figure V-4. Secreted and Intracellular M-T2 C-terminal Truncated Proteins are Expressed in Cells Infected with Various Recombinant Vaccinia Virus Constructs

A. Secreted protein harvested from supernatants were concentrated by precipitation with methanol:acetone (1:1), and analyzed by Western analysis, probing with anti-MT2 antibody. Supernatants from VV-601 (lane 1), VV-MT2-infected cells (lane 2), and the different M-T2 truncations ΔL113 (lane 3), ΔE174 (lane 4), ΔY236 (lane 5), ΔI249 (lane 6), ΔI260 (lane 7), ΔI279 (lane 8), ΔD303 (lane 9) and ΔN169 (lane 10). Size standards (83, 50.6, 35.5, and 29.1 kDa) are indicated.

B. Cell lysates from infected cells were analyzed by Western analysis, probing with anti-MT2 antibody. Intracellular protein from VV-601 (lane 10) and VV-MT2-infected cells (lane 1) and the different M-T2 truncations ΔL113 (lane 2), ΔE174 (lane 3), ΔY236 (lane 4), ΔI249 (lane 5), ΔI260 (lane 6), ΔI279 (lane 7), ΔD303 (lane 8)and ΔN169 (lane 9). M-T2 variants were first immunoprecipitated with anti-MT2 antibody before Western analysis. Immunoprecipitations from VV-601-infected cells (lane 11), infected cells expressing ΔL113 (lane 12), ΔE174 (lane 13) and ΔN169 (lane 14). Size standards (101, 83, 50.6, 35.5, 29.1, and 20.1 kDa) are indicated.

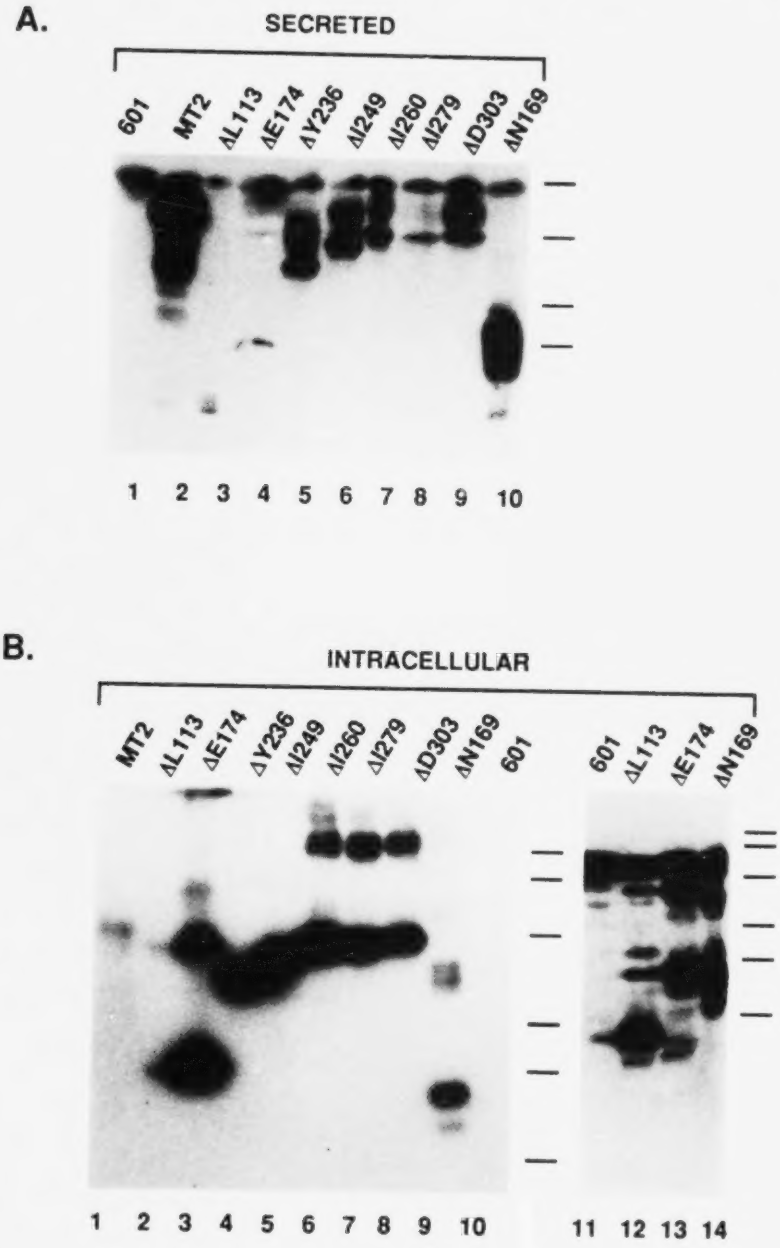

A.
SECRETED
601
MT2
ΔL113
ΔE174
ΔY236
ΔI249
ΔI260
ΔI279
ΔD303
ΔN169
1 2 3 4 5 6 7 8 9 10
B.
INTRACELLULAR
MT2
ΔL113
ΔE174
ΔY236
ΔI249
ΔI260
ΔI279
ΔD303
ΔN169
601
601
ΔL113
ΔE174
ΔN169
1 2 3 4 5 6 7 8 9 10
11 12 13 14

Figure V-5. Structure of M-T2 Proteins Containing CRD Deletions

The M-T2 CRD deletion proteins are outlined, with ovals representing the four CRDs, asterisks denoting predicted N-glycosylation sites, black boxes designating the N-terminal leader sequence and arrowheads indicating leader cleavage sites.

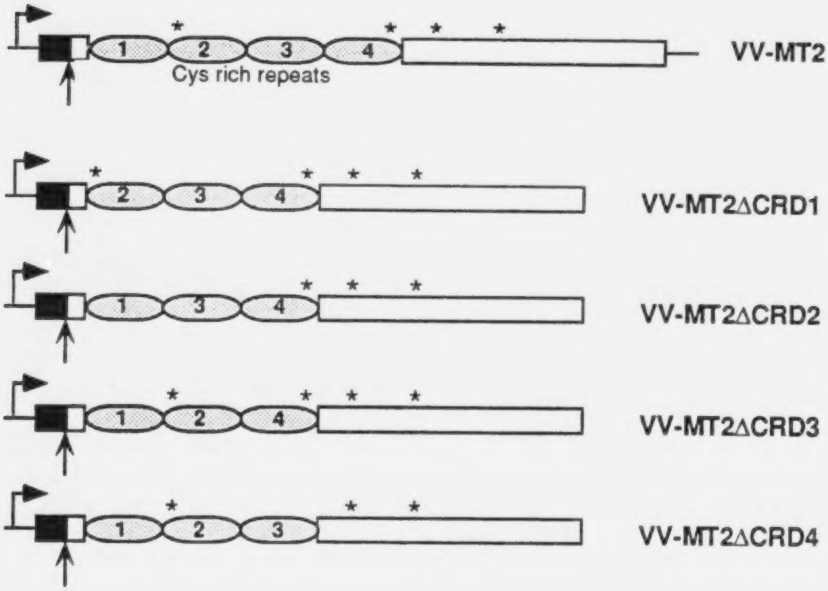

Cys rich repeats
VV-MT2
VV-MT2ΔCRD1
VV-MT2ΔCRD2
VV-MT2ΔCRD3
VV-MT2ΔCRD4

Figure V-6. Analysis of M-T2 Proteins Containing CRD Deletions

BGMK cells were infected with the different recombinant vaccinia viruses expressing CRD deletions in the presence of [^{35}S]met/cys, 6-22 hours post-infection, and M-T2 was immunoprecipitated from extracellular supernatants (B) or cell lysates (C). Proteins that immunoprecipitate with anti-MT2 antibody are displayed for control VV-601 (lane 1), VV-MT2 (lane 2) or viruses expressing ΔCRD1 (lane 3), ΔCRD2 (lane 4), ΔCRD3 (lane 5), and ΔCRD4 (lane 6). Size standards (101, 83, 50.6, 35.5, 29.1, and 20.1 kDa) are indicated.

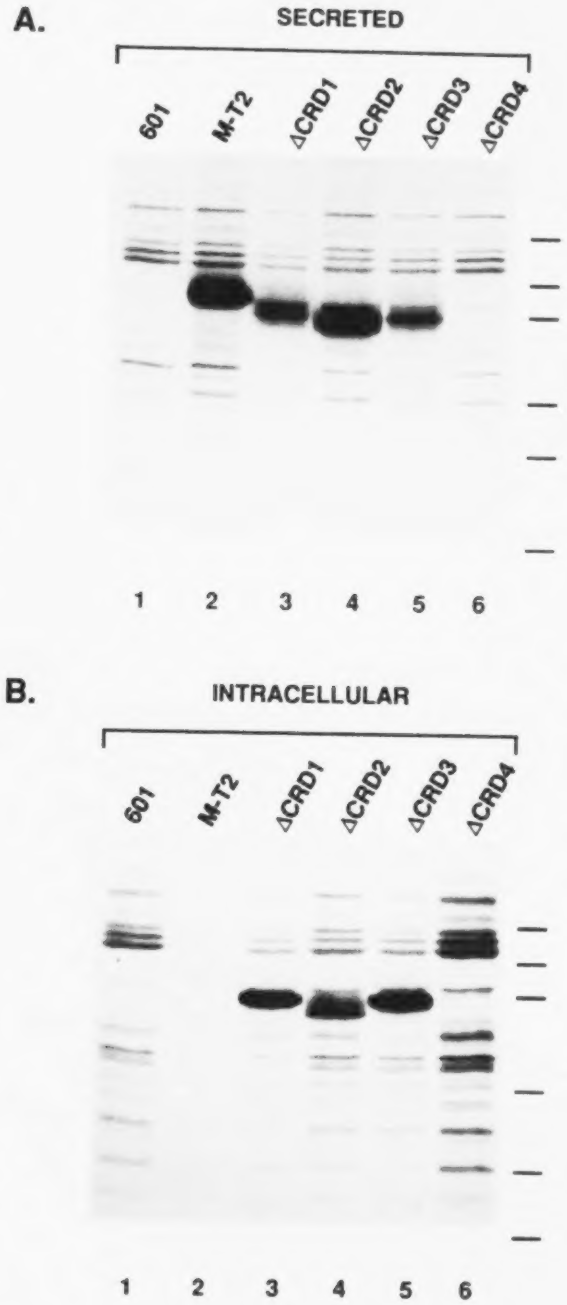

A.
SECRETED
601
M-T2
ΔCRD1
ΔCRD2
ΔCRD3
ΔCRD4
1
2
3
4
5
6
B.
INTRACELLULAR
601
M-T2
ΔCRD1
ΔCRD2
ΔCRD3
ΔCRD4
1
2
3
4
5
6

Figure V-7. Co-Immunoprecipitation of Rabbit TNF with M-T2 Deletion Variants

[^{35}S]-met/cys labelled rabbit TNF was combined with either secreted proteins (panel A) or cell-associated proteins (panel B) derived from cells infected with the different VV-MT2 constructs, and immunoprecipitated with anti-MT2 antibody as described under Materials and Methods. Panel A. Radiolabelled rabbit TNF was immunoprecipitated with anti-rabbit TNF antibody (lane 1) or co-immunoprecipitated with anti-MT2 antibody and supernatants from cells infected with VV-601 (lane 2), VV-MT2 (lane 3), or supernatants containing ΔL113 (lane 4), ΔE174 (lane 5), ΔY236 (lane 6), ΔI260 (lane 7), ΔD303 (lane 8), ΔCRD1 (lane 9), ΔCRD2 (lane 10) and ΔCRD3 (lane 11). Panel B. Radiolabelled rabbit TNF was immunoprecipitated with anti-rabbit TNF antibody (lane 1) and co-immunoprecipitated with anti-MT2 antibody alone (lane 2) or after mixing with lysates from cells mock-infected (lane 3) or infected with VV-MT2 (lane 4), VV-601 (lane 5), or lysates containing ΔL113 (lane 6), ΔN169 (lane 7), ΔE174 (lane 8), ΔY236 (lane 9), ΔI260 (lane 10) ΔI279 (lane 11) and ΔD303 (lane 12).

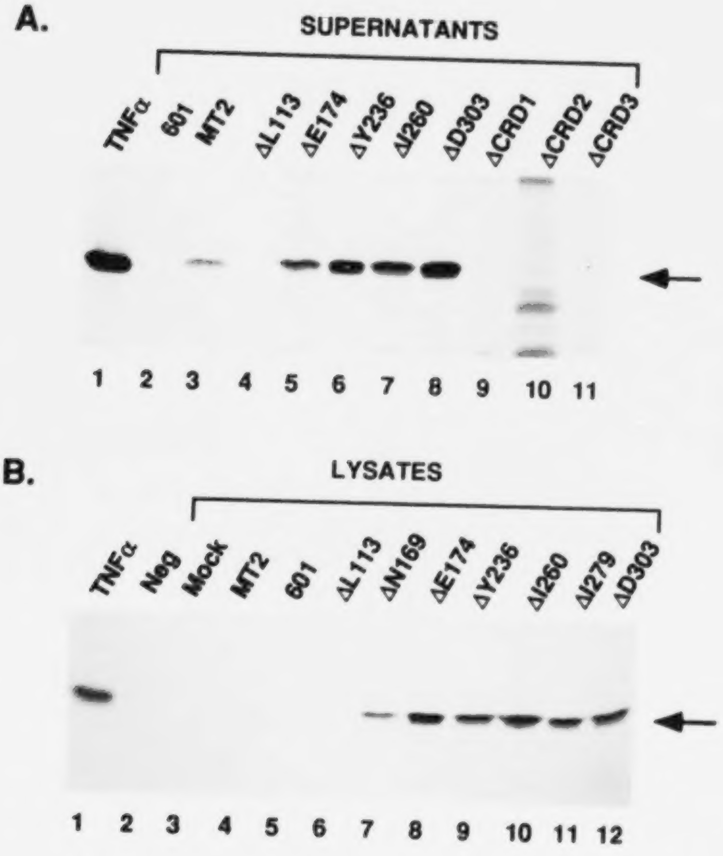

A.
SUPERNATANTS
TNFα
601
MT2
ΔL113
ΔE174
ΔY236
ΔI260
ΔD303
ΔCRD1
ΔCRD2
ΔCRD3
1 2 3 4 5 6 7 8 9 10 11
B.
LYSATES
TNFα
Neg
Mock
MT2
601
ΔL113
ΔN169
ΔE174
ΔY236
ΔI260
ΔI279
ΔD303
1 2 3 4 5 6 7 8 9 10 11 12

Figure V-8. Inhibition of Rabbit TNF-Induced Cytolysis of L929-8 Cells by the M-T2 Variants

A. Actinomycin-D sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF alone or together with 500 ng/ml purified M-T2 protein, supernatant containing 500 ng/ml ΔN169, ΔE174, ΔY236, ΔI260, and ΔD303, or 100 ng/ml ΔL113. B. Actinomycin-D sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF alone or together with 500 ng/ml purified M-T2 protein, or 500 ng/ml ΔCRD1, ΔCRD2, and ΔCRD3.

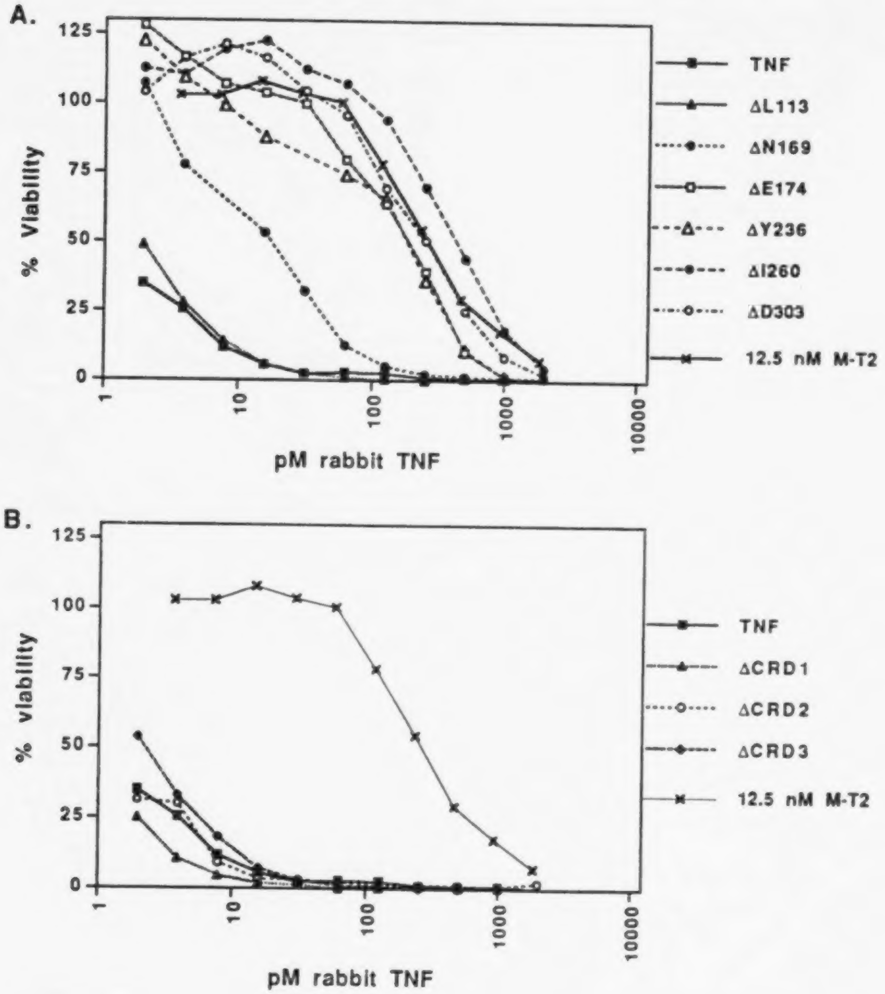

A.
% Viability
125
100
75
50
25
0
1
10
100
1000
10000
pM rabbit TNF
TNF
ΔL113
ΔN169
ΔE174
ΔY236
ΔI260
ΔD303
12.5 nM M-T2
B.
% viability
125
100
75
50
25
0
1
10
100
1000
10000
pM rabbit TNF
TNF
ΔCRD1
ΔCRD2
ΔCRD3
12.5 nM M-T2

TAble V-1. Observed SDS-PAGE Mobilities of M-T2 Variant Proteins

Variant	Predicted Size, kDa	Apparent Size, kDa		Endo H$_f$ Sensitivity
		Secreted	Intracellular	
MT2	33.4	55-65, 28-50*	58	+
ΔL113	10.1	13	10-13	N.D.[1]
ΔN169	16.3	14.5-26*	20-22, 43	+
ΔE174	17.3	19	22-28, 47-56	+
ΔY236	23.1	33-53*	38-47	+
ΔI249	24.9	36-59*	45-53	+
ΔI260	26.5	40-66*	47-58, 88-96	+
ΔI279	28.0	40-66*	47-58, 88-96	+
ΔD303	30.4	40-66*	47-58, 88-96	+
ΔCRD1	28.8	58-62	55-59	N.D.
ΔCRD2	28.8	53-61	54-57	N.D.
ΔCRD3	29.0	58-60	53-57	N.D.
ΔCRD4	28.2			

*Multiple bands observed

[1] N.D., not determined

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CHAPTER VI - DISTINCT DOMAINS OF M-T2 MEDIATE EXTRA-CELLULAR AND INTRACELLULAR ANTI-IMMUNE RESPONSES

INTRODUCTION

Myxoma-virus replication in lymphoid cells is thought to be the principle mechanism by which the virus is able to disseminate via the lymphoreticular system and establish infections at secondary sites within the host, and is an important determinant for the extreme virulence of the virus in infected rabbits (1). Two myxoma proteins, M-T2 and M11L have been shown to be involved in the inhibition of apoptosis in virus-infected RL-5 cells (CD4$^+$-T cell lymphoma), and are required for viral replication within these cells (2). The M11L gene product has also been shown to be important for viral replication within primary rabbit splenocytes (3). Viruses in which the M11L or M-T2 genes are inactivated are attenuated within rabbits (4), and with the M-T2 inactivated virus, there was a reduction in the number and severity of lesions at secondary sites (5). These different gene products may thus be important for viral replication within and dissemination via subpopulations of rabbit lymphoid cells.

Thus, the dual ability of M-T2 protein to inhibit extracellular TNF as well as prevent apoptosis of infected lymphocytes may both be important factors in virus pathogenesis. Although M-T2 protein was first characterized as a secreted viroprotein (5, 6), the anti-apoptotic activity of M-T2 protein could not be replicated from an extracellular location (2). Here we investigate the expression of M-T2 protein in virus-infected cells and study its inhibitory properties. We report the discovery of a cell surface ligand, distinct from TNF, located on the cell membrane of RL-5 cells that can be inhibited by extracellular M-T2 protein. We have also tested a series of recombinant myxoma virus constructs that express nested C-terminal truncation of M-T2 for their ability to inhibit apoptosis from an intracellular location in virus-infected lymphocytes.

RESULTS

Kinetics of M-T2 Synthesis and Secretion from Myxoma-Infected Cells

In addition to TNF inhibition, M-T2 protein also plays an anti-apoptotic role in viral infection. For example, RL-5 cells (CD4$^+$-T cells) infected with the T2-inactivated virus, vMyxt2g, but not the parental virus expressing full length M-T2, vMyxlac, undergo a rapid apoptotic response that precludes productive virus replication (2). The effect of M-T2 appears to be from an intracellular location because exogenous purified M-T2 protein cannot protect against the apoptosis induced by vMyxt2g. Moreover, cell-associated and secreted M-T2 protein could be detected within myxoma-infected BGMK and RL-5 cell lysates and supernatants throughout infection (data not shown). To determine when during viral infection M-T2 protein was synthesized and processed, BGMK cells were infected with myxoma, and at various times post-infection, newly synthesized protein was labelled with [^{35}S] met/cys for the 2 hour interval immediately prior to harvest. Immunoprecipitations from mock-or v-Myxt2g-infected cells, labelled for 4 hours revealed the absence of background proteins immunoprecipitating with the M-T2 antibody (Fig. VI-1A, lanes 1, 2). Newly synthesized M-T2 protein was detected at each 2 hour time interval throughout a 24 hour round of infection, indicating M-T2 mRNA was translated throughout the virus infection (Fig. VI-1A, lanes 2-14).

To investigate M-T2 secretion, vMyxlac-infected BGMK cells were cultured continuously in the presence of [^{35}S] met/cys label, and supernatants collected at 2-hour intervals. Secreted M-T2 protein was first detected in the supernatants collected between 2-4 hours post infection (Fig. VI-1B, lane 4). In each subsequent 2 hour interval, up to 12 hours post-infection, labelled M-T2 protein was secreted from infected cells (lanes 4-8). After 12 hours of infection, M-T2 protein secretion ceased although it was being synthesized within the cell (see Fig. VI-1 A and B, lanes 9-14).

We tested whether the rate at which synthesized M-T2 protein was secreted varied during infection by pulse-chase analysis. As seen in Figure VI-1C, pulse-labelled M-T2 protein was synthesized in vMyxlac-infected BGMK cells within a 30 minute pulse beginning 2 hours post-infection (lane 3), but rapidly disappeared from the cell within chase periods of over 1 hour (lanes 6-8) and was detected in the supernanant fractions (lanes 9-12). By two hours post-label, most of the protein had been secreted (lane 10), and very little intracellular M-T2 was detected (lane 6). In contrast, when cells were pulsed with [^{35}S] met/cys only 2 hours later, i.e. 4 hours post-infection, it took much longer for the M-T2 protein produced to be secreted into the supernatant (Fig. VI-1D, lanes 3-11). For example, after a 2 hour chase period, approximately half of the labelled M-T2 had been secreted (lanes 3 and 4), whereas the same 2 hour chase period performed only 2 hours earlier resulted in almost quantitative secretion (compare with lane 6 in Fig. VI-1C). Thus, within the first four hours of infection, the rate at which M-T2 protein was secreted was dramatically altered, suggesting that other viral proteins might be altering M-T2 trafficking and/or secretion as the infection proceeds.

Intracellular M-T2 is resistant to digestion with Endo H

Endoglycosidase H (Endo H) is an enzyme which removes N-linked oligosaccharide chains from immature glycoproteins as found in the endoplasmic reticulum and cis-Golgi compartments. As the glycoproteins travel through the normal secretory pathways of the cell, processing by α-mannosidase II in the medial Golgi renders the glycoproteins progressively resistant to digestion by Endo H. Thus, sensitivity to digestion by Endo H is, in general, a marker for glycoproteins that have not yet entered the medial Golgi or later compartments. To study the trafficking of M-T2 protein, the Endo H sensitivity of ^{35}S-labelled M-T2 protein, produced in myxoma-infected BGMK and RL-5 cells during a 30 minute pulse with [^{35}S]met/cys was examined after varying amounts of chase time. In lysates from BGMK cells harvested immediately after the pulse, a 47 kDa Endo H sensitive form of M-T2 protein was observed (Fig. VI-2A, compare lanes 5 and 6), that was absent as expected from mock and vMyxt2g-infected cells (lanes 1-4). The amount of intracellular labelled M-T2 protein decreased with time of chase: Endo H sensitive-intracellular M-T2 protein was observed at 30 minutes, 1 hour and 2 hours post label (lanes 7-12), but had disappeared by the 3 hour chase time (lanes 13-14). Surprisingly, the M-T2 protein present within the cell could not be chased to an Endo H resistant form, indicating intracellular M-T2 had not been modified by α-mannosidase II present in the medial and trans Golgi, and processing to Endo H resistance was in fact the terminal modification before M-T2 secretion.

Figure VI-2B shows a pulse chase experiment with infected T lymphocytes (RL-5 cells), pulsed with [^{35}S] met/cys at 2 hours post infection. M-T2 protein, migrating at 57 kDa and 47 kDa, with and without Endo H treatment, was observed after the 30 minute pulse in vMyxlac-infected cells, but not in mock or vMyxt2g-infected cells (Fig. VI-2B, lanes 1-6). The intensity of the M-T2 signal within infected cells was gradually reduced over a 5 hour period (lanes 7-16), but intracellular ^{35}S-labelled M-T2 persisted even after a 5 hour chase and the majority of the protein remained endo H sensitive throughout the 5 hour chase, indicating that M-T2 was being secreted from RL-5 cells at a slower rate compared to BGMK cells. Moreover, this slower rate of M-T2 secretion from RL-5 cells could be consistently repeated. Unlike the results with BGMK cells, however, a higher molecular weight diffuse species, migrating between 58-64 kDa, was observed after a 1 hour chase, which gradually disappeared from the cells and by 5 hours was totally absent. This species appeared to be moderately resistant to Endo H digestion, as a residual faint diffuse band persisted after Endo H digestion. To investigate this unexpected aspect of M-T2 processing, RL-5 cells were infected with vMyxt2g or vMyxlac, in the presence of [^{35}S] met/cys for 16 hours to uniformly label the cell-associated pool of M-T2 and the accumulated secreted forms. M-T2 protein was then immunoprecipitated from supernatants and cell lysates, digested with Endo H, and resolved by SDS-PAGE (Fig. VI-2C). In cell lysates, harvested 16 hours post-infection, two forms of M-T2 protein were immunoprecipitated, a sharp 56 kDa band and a diffuse 59-66 kDa band (lane 3). Upon digestion with Endo H, both forms were reduced to a 46 kDa species, with some of the 59-66 kDa protein Endo H resistant (lane 4). Both species were also observed within supernatants; however, after secretion, the 59-66 kDa species remained Endo H resistant, whereas the 56 kDa species was Endo H sensitive (lanes 7-8). Thus, it appears that the RL-5 cells secreted two forms of M-T2 protein. Some of M-T2 secreted was Endo H resistant, typical of proteins secreted through the Golgi network, but a significant proportion of M-T2 remained Endo H sensitive, which was either not modified to a mature glycoprotein within the Golgi or was being secreted via another pathway.

Figure VI-2D shows the Endo H sensitivity of M-T2 protein secreted from vMyxlac infected BGMK cells by 4 hours post-infection. Upon digestion with Endo H, the 56 kDa secreted M-T2 was reduced to 56 kDa, 53 kDa, 49 kDa and 46 kDa bands (compare lanes 5 and 6). Since there are four potential N-glycosylation sites within M-T2 protein, this result suggests that the secreted M-T2 was only partially processed to Endo H resistant forms during passage through the cell. Alternatively, M-T2 protein was processed to both hybrid and complex oligosaccharide chains, of which only the complex chains were resistant to Endo H (7). It is unlikely that specific N-glycosylation sites within M-T2 were resistant to modification because of protein conformation or interactions with other proteins because then only one partially endo H resistant species would have been observed.

Because of these surprising results showing Endo H sensitive, secreted M-T2, we examined another secreted myxoma protein, M-T7 (the interferon-γ receptor homolog), to determine if it was Endo-H sensitive. Figure VI-2E shows M-T7 protein immunoprecipitated from supernatants of vMyx-lac-infected BGMK cells (lanes 3-6) or RL-5 cells (lanes 9-12), compared to controls from supernatants from mock-infected cells (lanes 1, 2, 7 and 8). The majority of M-T7 protein secreted from infected BGMK cells harvested at 4 and 8 hours post-infection was Endo H sensitive, although a faint amount of Endo H resistant protein was observed (lanes 4 and 6). In contrast, the majority of M-T7 secreted from RL-5 cells was completely Endo H resistant (lanes 10 and 12). Thus, like M-T2, M-T7 was secreted from infected cells as both Endo H sensitive and Endo H resistant glycoforms, the proportion of each glycoforms varying depending on the cell line infected, but unlike M-T2, M-T7 was fully processed to Endo-H resistant forms in infected RL-5 cells.

Northern analysis of M-T2 gene expression

Since M-T2 protein was being synthesized at both early and late times of infection, we investigated whether M-T2 message levels were present at these times by Northern analysis. DNA sequence immediately upstream of the M-T2 open reading frame (orf) shows all the important features of an early poxviral gene, expressed prior to viral DNA replication. Moreover, this sequence is very similar to the sequence immediately 5' of the T2 orf in the closely related leporipoxvirus, Shope fibroma virus, *S-T2*, which has been shown to be regulated as an early gene (5, 8). However, an unusual array of nine related tandem repeats, 12-20 nucleotides long, is present upstream of the M-T2, between nucleotides -52 and -182, which is absent in the SFV sequence (5), which could affect promoter function of M-T2. Figure VI-3A shows a Northern blot of RNA prepared from myxoma virus infected BGMK cells, and probed with a 0.9 kb PCR product of M-T2 coding sequence. Lanes 2 and 6 show the absence of stable message from cells infected with the M-T2 inactivated virus, vMyxt2g. However, a 2.9 kb message was detected by 2 hours in vMyxlac-infected cells (lane 3), which was still present at 12 hours when the cells were treated with arabinosyl cytosine (araC), an inhibitor of DNA synthesis, confirming that *M-T2* was being expressed as an early gene (lane 4). By 12 hours post-infection, the early 2.9 kb transcripts could hardly be detected, even though M-T2 protein was continuously synthesized throughout the infectious cycle. Instead, the M-T2 probe hybridized to messages of 4.9, 3.3 and 2.1 kb from vMyxlac-infected cells, but not similar sized transcripts from vMyxt2g-infected cells (lanes 5 and 6). These multiple bands resembled the run-on transcripts observed with gene transcribed at late times of poxviral infection when 3' terminations of viral mRNA become more random in nature (9). In Figure VI-3B, showing a Northern blot of RNA prepared from cells infected with vMyxlac and harvested at various times, the 2.9 kb message was the only message observed hybridizing with the M-T2 probe at 3 hours post-infection (lane 1); by 6 hours, the amount of 2.9 kb message had dropped dramatically, but the 4.9, 3.3 and 2.1 kb M-T2 transcripts instead predominated (lane 2). Confirmation of the origin of these mRNA will require rigorous analysis of their 5' ends by primer extension or nuclease S1 analysis to confirm whether they are expressed late from the M-T2 promoter, but the protein labelling experiments (Fig. VI-2) indicate that M-T2 translation continues well into late times of infection.

Growth of RL-5 cells is not sensitive to rabbit TNF

Since M-T2 protein specifically binds and inhibits rabbit TNF, and TNF has been shown to induce cytolysis/apoptosis of many different cells (10-12), we investigated whether TNF is capable of inducing apoptosis in infected RL-5 cells. We first examined whether RL-5 cells were sensitive to growth inhibition by TNF. RL-5 cells were cultured for 5 days in the presence or absence of 50 nM rabbit TNF, a concentration found to be completely cytostatic for rabbit TNF–sensitive RK-13 and SIRC rabbit cell lines (Chapter III), but no difference between the growth of RL-5 cells in the presence or absence of rabbit TNF was observed (Fig. VI-4), indicating that rabbit TNF does not have a cytostatic effect on RL-5 cells. We also tested to see whether TNF had a cytolytic effect on RL-5 cells in the presence of inhibitors of protein synthesis; however, treatment with protein synthesis inhibitors alone induced apoptosis in RL-5 cells (data not shown).

We investigated to see whether RL-5 cells could be induced to secrete TNF. RL-5 cells were cultured overnight, treated with LPS, 12 hours, or infected with vMyxlac, vMyxt2g, or vaccinia virus, VV-601, 18 hours. Supernatants were concentrated ten-fold, and then analyzed for secreted rabbit TNF by cytolysis of L929-8 cells (13). As shown in Table 1, no secreted TNF was observed in supernatants from RL-5 cells, LPS-stimulated RL-5 cells, or RL-5 cells infected with myxoma or vaccinia virus. In contrast, rabbit peripheral blood lymphocytes (PBLs), enriched for monocytes, could be stimulated with LPS to secreted TNF. Thus, RL-5 cells do not appear to secrete rabbit TNF.

Supernatants and lysates prepared from RL-5 cells were also examined for the presence of TNF by Western blotting, or radiolabelling cells and immunoprecipitating with an anti-rabbit TNF antibody. Rabbit TNF could not be detected within RL-5 lysates or supernatants by either of these methods (data not shown), although we cannot rule out the possibility of a sensitivity problem in that there may not have been enough TNF protein present to be detected by these methods.

M-T2 Protein Blocks the Cell-Cell Cytotoxic Effects of RL-5 Cells

Although RL-5 cells do not secrete or respond to rabbit TNF, they might express a transmembrane form of TNF which could be detected by cell-contact mediated cytolysis of TNF-sensitive cells. Adherent, actinomycin-D sensitized L929-8 cells were overlayed with increasing numbers of non-adherent RL-5 cells, and after 18 hours, L929-8 cell viability monitored by neutral red-uptake. As seen in Figure VI-5, contact with RL-5 cells was directly cytotoxic to L929-8 cells, and this cytotoxic effect could be abolished by titrating out the RL-5/L929-8 cell ratio. Moreover, a direct protective effect was observed when the L929-8 cells were overlayed with RL-5 cells in the presence of 100 nM purified M-T2 protein. An increase in L929-8 cell viability from 4% to 30% was observed at an RL-5:L929-8 ratio of 15:1, in the presence of exogenous M-T2 protein, previously shown to protect against TNF-induced cytolysis of L929-8 cells. Surprisingly, no protection with anti-rabbit TNF antibody, was observed at any of the RL-5:L929-8 ratios tested (squares), even though the anti-rabbit TNF antibody was an effective inhibitor of 18 kDa TNF ligand-induced cytolysis on L929-8 cells (inset). The fact that M-T2 protein but not anti-rabbit TNF antibody protected L929-8 cells from cytolysis induced by contact with RL-5 cells, and the inability to observe any secreted or intracellular form of TNF protein from RL-5 cells suggests that this new M-T2-inhibitable factor may be another cell-surface member of the TNF superfamily, capable of cytotoxicity, such as the lymphotoxin α/β complex, CD40 ligand or Fas ligand.

Construction of Myxoma viruses expressing C-terminal deletions

In Chapter V, the construction of a series of vaccinia viruses expressing C-terminal truncations of M-T2 protein was described, and it was demonstrated that only the 3 N-terminal cysteine-rich domains (CRDs) are required to bind and inhibit rabbit TNF. A series of recombinant myxoma viruses, expressing these M-T2 truncations, was constructed by homologous recombination into the *M-T2* loci of vMyxlac, using gpt as a dominant selection marker. The various myxoma viruses used in this paper are outlined in Figure VI-6A. Full length M-T2 protein is shown, outlining the CRD region, cysteine residues within the peptide sequence, the N-terminal signal cleavage after G16, and four potential N-glycosylation sites at N66, N181, N205 and N238. The deletion mutants begin with ΔD303, in which only the C-terminal 24 amino acids have been removed, and end with ΔL113, with only the N-terminal 34% of M-T2 protein sequence present, and are named according to the first amino acid deleted within the truncation (Figure VI-6B). The deletions, ΔL113, ΔN169 and ΔE174 are all within the CRDs; ΔL113 contains 19% of the third CRD, and ΔN169 and ΔE174 contain 47 and 57% of the fourth repeat, respectively. All of the other deletions contain truncations beginning after the N-terminal CRDs. Note that although the vMyxt2g knockout virus could potentially express the N-terminal 45 amino acids of M-T2, no mRNA was detected for this sequence in infected cells (Fig. VI-3), and no immunoreactive fragments of M-T2 protein could be detected (data not shown).

All of the C-Terminal Truncations Except ΔL113 Co-Immunoprecipitate Rabbit TNF

To determine whether the various myxoma-expressed M-T2 truncations are capable of interacting with rabbit TNF, the ability of the M-T2 truncations to co-immunoprecipitate rabbit TNF was tested. Radiolabelled rabbit TNF was prepared by infecting cells with VV-T2RaTNF in the presence of [^{35}S] met/cys and collecting the supernatants. Total proteins from cells infected with the myxoma viruses containing the different C-terminal M-T2 deletions were combined with radiolabelled rabbit TNF, and were then immunoprecipitated with affinity purified anti-MT2 antibody. In Figure VI-7, lane 1, 18 kDa rabbit TNF secreted from VV-T2RaTNF infected cells was immunoprecipitated with a polyclonal anti-rabbit TNF antibody and serves as a size marker for rabbit TNF. Rabbit TNF was not immunoprecipitated by the affinity purified anti-MT2 antibody because no rabbit TNF could be detected when it was combined with proteins from vMyxt2g-infected cells (lane 11). However, rabbit TNF was co-immunoprecipitated, as expected, with wild-type M-T2 protein from vMyxlac-infected cells (lane 2). The different C-terminal truncations, ΔN169 to ΔD303, expressed from myxoma virus, were all able to co-immunoprecipitate rabbit TNF (4-10) in a fashion identical to the equivalent C-terminal truncations expressed from vaccinia virus vectors. ΔL113, however did not coimmunoprecipitate with rabbit TNF (lane 3). The ΔL113 truncation begins within the third repeat and only expresses 8 amino acids of this repeat. The inability of ΔL113 to co-immunoprecipitate rabbit TNF is completely in agreement with the results from vaccinia virus expressed trunctions and deletions within M-T2 showing that intact copies of the first three CRDs at the N-Terminus is critical for TNF binding and inhibition.

Growth of the Myxoma Variants Bearing C-Terminal Truncations in BGMK and RL-5 cells

Previously, it was shown that RL-5 cells readily undergo apoptosis when infected with vMyxt2g, the T2-inactivated virus, but not when infected with vMyxlac, the parental virus for the vMyxt2g construction (Macen *et al.*, 1996). Moreover, the replication of vMyxt2g in RL-5 cells was reduced compared to vMyxlac, indicating that expression of M-T2 is important for viral propagation within RL-5 cells. Therefore, we investigated whether the growth of any of the M-T2 truncation viruses was attentuated in RL-5 cells. Figure VI-8A shows the 36-hour virus yields from RL-5 cells infected with vMyxlac, vMyxt2g, vMyx(wt:strain Lausanne), vMyxt2r (M-T2 revertant of vMyxt2g), and the different myxoma M-T2 C-terminal truncation viruses, expressed as the output yield/input virus. Although even the wild-type myxoma virus yield is lower in RL-5 cells than BGMK cells by an order of magnitude, the growth of vMyxt2g was clearly attentuated in RL-5 cells, compared to the virus yield from vMyxt2r, indicating that restriction of vMyxt2g growth in RL-5 cells is due to the disruption in M-T2. When the replication of myxoma viruses expressing each of the C-terminal truncations was examined, surprisingly they all replicated within RL-5 cells to an extent comparable to vMyxlac, indicating that all of the M-T2 truncated proteins expressed inhibited the apoptotic effect. Interesting, the myxoma virus expressing the ΔL113 truncation, which does not co-immunoprecipitate rabbit TNF nor protect against TNF-induced cytolysis, was able to replicate to levels comparable with vMyxlac in RL-5 cells. This data further supports the notion that the attentuation of the growth of vMyxt2g in RL-5 cells was not due to any TNF-induced cytolysis of infected cells. Moreover, when RL-5 cells were infected with vMyxt2g or vMyxlac in the presence of 50 nM rabbit TNF, in vast excess over the amount of M-T2 protein secreted from infected cells, no difference was observed between virus yields when rabbit TNF was present or absent.

The growth of the different viruses was also examined in permissive BGMK cells, in which the vMyxt2g virus replicates normally compared to vMyx(wt). As expected, virus yields from cells infected with the different truncations were all comparable, within experimental error, as expected (Fig. VI-8B).

Infection with vMyxt2g, but not with Myxoma Viruses Expressing C-Terminal Deletions in M-T2, Induces Apoptosis in RL-5 cells

RL-5 cells were infected for 10 hours with the various myxoma viruses and examined for the induction of apoptosis. DNA fragmentation within RL-5 cells, a characteristic feature of apoptosis, was measured by TdT-incorporation of fluorescein-12-dUPT into DNA and quantitated by flow cytometric analysis. As previously shown, infection of RL-5 cells with vMyxt2g revealed a significant population of cells undergoing apoptosis (39%, Fig. VI-9C), whereas this population was not observed with mock or vMyxlac infection (Fig. VI-9 A,B). When RL-5 cells were infected with vMyxt2g in the presence of 10 nM purified M-T2 protein, a comparable proportion of the cells (37%) were still found to be apoptotic (Fig. VI-9D). As a check to ensure that the M-T2 protein was still active, the media containing the purified M-T2 protein, was tested before and after culturing with the vMyxt2g-infected RL-5 cells, and the ability of the M-T2 protein to inhibit TNF-induced cytolysis of L929-8 cells did not change (Fig. VI-9M).

RL-5 cells were also infected with the myxoma viruses expressing the various C-terminal truncations. Infection with each of these mutant viruses, including vMyxMT2ΔL113, did not induce any significant apoptosis within RL-5 cells (Fig. VI-9 D-L). This result is in agreement with the previous observation that these viruses were able to replicate within RL-5 cells comparable to vMyxlac (Fig. VI-8A). Note that secreted forms of the various deletions were not observed, and exogenous full length M-T2 protein was unable to protect vMyxt2g-infected cells from undergoing apoptosis. Thus, all of these results confirm the contention that M-T2 plays an anti-apoptotic role from an intracellular location. Importantly, ΔL113 does not even contain a functional

TNF-binding domain, yet expression of this mutant protein fully protects virus-infected RL-5 cells from undergoing apoptosis. We conclude that the anti-TNF properties of secreted M-T2 are distinct from the anti-apoptotic effects of the intracellular form of the same protein.

DISCUSSION

According to the original model of M-T2 action for myxoma virus virulence, M-T2 protein, secreted from infected cells, binds and sequesters the TNF produced and secreted locally by immune cells in response to viral infection (5, 14). By preventing ligand triggering of TNFRs, M-T2 protein would inhibit both direct TNF cytolysis of infected cells and other secondary immune responses dependent upon TNF. In support of this model, we have shown that secreted M-T2 protein binds rabbit TNF in the pM range with an affinity comparable to that of TNF with cellular receptors, and inhibits the cytolytic effect of rabbit TNF at molar ratios similar to that observed with soluble versions of the TNFRs (Chapter IV). Thus, it was unexpected when the ability of M-T2 to inhibit the apoptosis of myxoma-infected RL-5 cells could not be mimmicked by the addition of exogenous, purified M-T2 at concentrations in vast excess over that required to inhibit the cytolytic activity of rabbit TNF (2), suggesting, instead, that intracellular M-T2 protein was mediating the anti-apoptotic effect.

M-T2 expression and secretion was analyzed in BGMK and RL-5 cells. BGMK cells are permissive for growth of vMyxt2g but RL-5 cells are non-permissive due to the supervening apoptotic response to the virus infection Intracellular forms of M-T2 were readily detected at all times during infection in both BGMK and RL-5 cells, indicating that M-T2 protein would be at least available for binding to intracellular target protein(s). Although, M-T2 protein was continuously being synthesized throughout virus infection, the rate of M-T2 secretion, was significantly altered. Initially, M-T2 protein was rapidly processed and secreted, providing an extracellular source of M-T2 to inhibit local TNF present in the extracellular milieu of infected cells. By four hours post-infection, however, the rate of M-T2 secretion from BGMK cells had dropped dramatically and at later times, M-T2 protein was fully retained within myxoma-infected cells. This decrease could be due to the viral infection altering the host cell secretory machinery or it could be due to specific retention of M-T2 protein within infected cells.

In BGMK cell lysates, all the M-T2 protein observed in the steady state pools was sensitive to Endo H. Although M-T2 protein was secreted from these cells, intracellular M-T2 protein could not be chased to Endo H resistant or partially resistant species, indicating that for M-T2 protein, either processing to Endo H resistant forms was ineffecient or it was one of the last events to occur before secretion. However, M-T2 protein secreted from myxoma-infected BGMK cells was still mostly sensitive to digestion with Endo H, and the cleaved species resolved into at least four distinct species, indicating that both complex and hybrid or high mannose oligosaccharide units were attached at multiple N-glycosylation sites. The N-oligosaccharide chains present on secreted M-T2 protein had only been partially processed to complex oligosaccharide units, suggesting that myxoma infection may interfere with oligosaccharide maturation in BGMK cells.

In constrast, both Endo H sensitive and resistant forms of intracellular M-T2 protein were observed in cell lysates prepared from myxoma-infected RL-5 cells. All of the Endo H resistant M-T2 present within RL-5 cells could be chased to the culture supernatant, whereas, only some of the Endo H sensitive form was secreted during the chase times, indicating it was either being retained within the cell or secreted at a much slower rate. In light of the fact that M-T2 protein has an anti-apoptotic effect specifically in RL-5 cells, it is possible that the Endo H sensitive form may be retained within the cell through interaction with another protein in the apoptotic pathway. These two forms of M-T2 protein were also observed secreted from RL-5 cells. The majority of M-T2 protein in the secreted fraction was resistant to Endo H, indicating its oligosaccharide chains had been processed to mature, fully complex forms. A minor 56 kDa species was also secreted, which upon Endo H digestion reduced to a 46 kDa band, indicating none of its N-linked oligosaccharide units had been processed to complex sugar chains. This data suggests that M-T2 from RL-5 cells was trafficked via two different pathways; the majority of M-T2 glycoprotein secreted was processed to complex oligosaccharide units within the Golgi network, whereas a subpopulation of M-T2 did not encounter α-mannosidase II within the Golgi, presumably because M-T2 protein was complexed with another factor which either prevented oligosaccharide processing by the α-mannosidase II or diverted M-T2 into a distinct cellular compartment.

Differences were observed between BGMK and RL-5 cells both with the rates of M-T2 secretion and oligosaccharide processing. Completely Endo H resistant forms of M-T2 protein were observed in both cell lysates and supernatants from infected RL-5 cells but not BGMK cells, indicating that there are differences in oligosaccharide processing between the two cell lines. Variation in sugar processing can be expected between cells, and altering glycosylation is one mechanism to introduce variability and modify protein activities (15-17). These differences in oligosaccharide processing were not restricted to M-T2 protein. M-T7, the myxoma virus IFN-γ receptor homology, was also secreted from BGMK cells as one Endo H sensitive species, whereas from RL-5 cells, the N-linked oligosaccharides on M-T7 were processed to Endo H resistant complex chains before secretion. A close examination of glycosylation patterns might provide useful information on the intracellular trafficking of M-T2 protein within the cell. In RL-5 cells, the Endo H resistant and sensitive forms of M-T2 protein may have different functions in preventing infected cells from triggering the apoptotic response.

At the level of gene expression, we confirm here that M-T2 mRNA was detected both at early and late times of infection and in the presence of araC, a DNA synthesis inhibitor. At late times of infection, the message transcribed at early times which directed M-T2 protein synthesis vanished and was replaced by longer run-on transcripts. The corresponding T2 gene from the related SFV is also expressed at both early and late times of infection, as determined by S1 analysis of transcripts (8). The actual confirmation of the myxoma transcripts observed at late times as M-T2 transcripts, however, will require a rigorous analysis of their 5' ends. The indication that M-T2 transcription occurs at both early and late times, though, reaffirms the fact that nascent M-T2 protein is continuously expressed within the cell.

Although the M-T2 protein N-terminal domain shows homology with the CRDs of the TNFR superfamily, the C-terminal 132 amino acids do not show sequence homology with any cellular factor or unrelated viral protein. Each of the M-T2 C-terminal truncations, however, up to and including truncations within the third CRD, was able to fully protect myxoma-infected RL-5 cells from undergoing apoptosis, indicating the C-terminus was not involved in the anti-apoptotic response. The ΔL113 variant of M-T2, which only contains the first two CRDs and eight amino acids of the third CRD, was able to prevent apoptosis in RL-5 cells and to direct productive viral replication of myxoma virus, despite its inability to bind or inhibit TNF. Furthermore, all of the various M-T2 C-terminal truncations were retained as cell associated proteins, reinforcing the observation that it is intracellular M-T2 protein which inhibits viral-induced apoptosis while the extracellular form is responsible for TNF ligand inhibition.

Although it remains possible that M-T2 inhibits a downstream effector of an apoptotic signaling cascade, it seems more likely that M-T2, with its homology to the N-terminal domains of the TNFR family members, inhibits apoptotic signaling from a member of the TNFR superfamily. However, we can say that TNF, does not appear to mediate apoptosis within infected RL-5 cells. Since M-T2 protein binds and effectively inhibits rabbit TNF, we rigorously investigated TNF production and sensitivity in RL-5 cells. Rabbit TNF did not affect the growth of RL-5 cells in culture, it did not affect the replication of myxoma within infected RL-5 cells, and could not be detected secreted from RL-5 cells. Moreover, TNF could not be detected within RL-5 cell lysates by Western analysis and by immunoprecipitation (data not shown). Although RL-5 cells constitutively express a cell surface molecule that was cytotoxic to L929-8 cells, this surface factor does not appear to be membrane bound TNF in that inhibitory anti-rabbit TNF antibodies did not protect L929-8 cells from RL-5 mediated cell lysis, while extracellular M-T2 protein was an effective inhibitor of this cytotoxic ligand.

The RL-5 cell surface factor which was cytolytic to L929-8 cells could be a LTα/β heterotrimer. LTβ is another member of the TNF superfamily which forms complexes with LTα, anchoring it to the plasma membrane (18); the LTα/β heterotrimer binds to the LTβ receptor, a member of the TNFR family (19, 20). It is conceivable that M-T2 protein recognizes a LTα/β complex on RL-5 cells and prevents its association with a LTβ receptor on L929-8 cells. We cannot rule out the ligands for Fas or CD40 as mediators of RL-5 cell apoptosis. Viruses are known to have evolved mechanisms to specifically interfere with Fas signaling, such as the cowpox *crmA* gene (21). Infection of B and T lymphoblast cell lines with Vaccinia strain WR but not strain Copenhagen inhibited the Fas-mediated apoptosis of these cells (22).

We cannot rule out the possibility that M-T2 protein might associate with an intracellular apoptotic regulator or signaling molecule unrelated to the TNF or TNFR superfamilies. But at this point a more plausible rationale is that M-T2 protein may heterodimerize with a member of the TNFR superfamily. M-T2 protein is able to form disulphide-linked homodimers; if M-T2 protein could also form heterodimers with a member of the TNFR superfamily, M-T2 would interfere with receptor clustering and signaling by acting as a dominant-negative inhibitor. The membrane receptor/M-T2 complex could potentially still bind the appropriate ligand; however, as M-T2 protein lacks the appropriate intracellular signaling domains, the receptor/M-T2 complex would be unable to oligomerize and signal through the cytoplasmic death domain. C-terminal truncations of the p55 TNFR have been described which act in a dominant negative fashion to suppress signaling (23, 24). Moreover, a number of dominant interfering mutations within Fas have been described from individuals with autoimmune lymphoproliferative syndrome which impair Fas-mediated apoptosis (25). Under this model of M-T2 action, the ΔL113 truncation would have retained the ability to recognize and possibly form inhibitory heterodimers with a TNFR family member despite its inability to bind or inhibit the TNF ligand itself. Distinguishing between these hypothetical mechanisms of how intracellular M-T2 prevents the apoptotic response in infected lymphocytes will require cellular localization of M-T2 protein and identification of the cellular target proteins that associate with it.

Figure VI-1. Kinetics of M-T2 Protein Synthesis and Secretion in BGMK Cells

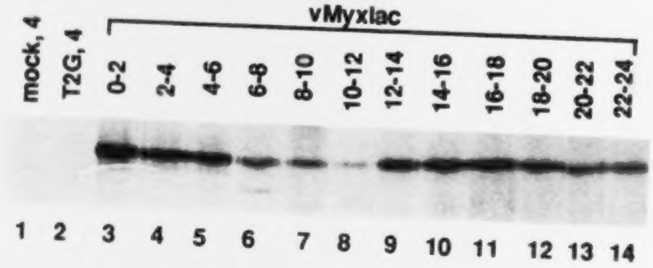
A. Kinetics of M-T2 synthesis during infection. Immunoprecipitations from mock- or vMyxt2g-infected BGMK cells infected and labelled with [^{35}S] met/cys for 4 hours (lanes 1, 2) and from vMyxlac-infected BGMK cells labelled with [^{35}S] met/cys 2 hours prior to harvest at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours post infection (lanes 3-14).

B. M-T2 protein secretion from infected BGMK cells. Immunoprecipitations from supernatants of mock-infected or vMyxt2g-infected BGMK cells infected and labelled with [^{35}S] met/cys for 4 hours (lanes 1, 2), or vMyxlac-infected BGMK cells continuously labelled with [^{35}S] met/cys throughout the infection, and the medium harvested and replaced at two hour intervals, harvesting at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours post-infection (lanes 3-14).

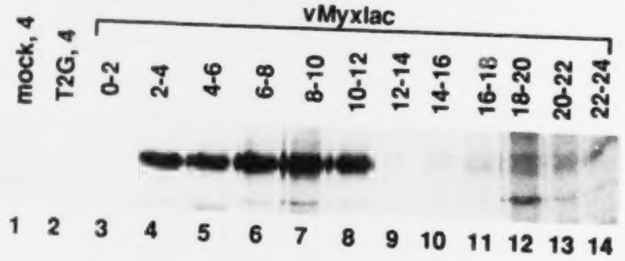
C. Pulse-chase analysis of M-T2 protein secretion from myxoma-infected BGMK cells synthesized at 2 hours post-infection. Infected BGMK cells were pulsed with [^{35}S] met/cys at 2 hours post-infection, and M-T2 protein immunoprecipitated from lysates of cells mock-, vMyxt2g-, or vMyxlac harvested 30 minutes later (lanes 1-3) or chased with cold medium for 30 min, 1, 2, 3 and 4 hours (lanes 4-8) or from supernatants of vMyxlac-infected cells chased with cold medium for 30 min, 1 and 3 hours (lanes 9-11).

D. Pulse-chase analysis of M-T2 protein secretion from myxoma-infected BGMK cells synthesized at 4 hours post infection. Infected BGMK cells were pulsed with [^{35}S] met/cys at 4 hours post-infection, and M-T2 protein immunoprecipitated from lysates of cells mock-, vMyxt2g-, or vMyxlac harvested 30 minutes later (lanes 1-3) or chased with cold medium for 2, 4, 6 and 8 hours (lanes 4-7) or from supernatants of vMyxlac-infected cells chased with cold medium for 2, 4, 6 and 8 (lanes 8-11).

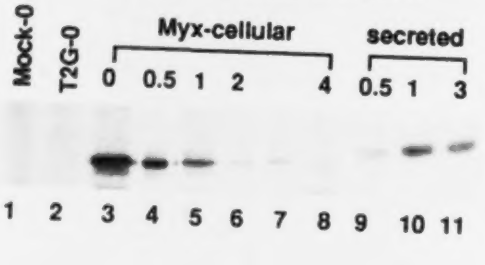
A. M-T2: BGMK INTRACELLULAR


vMyxlac
mock, 4
T2G, 4
0-2
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
20-22
22-24
1 2 3 4 5 6 7 8 9 10 11 12 13 14

B. M-T2: BGMK SECRETED


vMyxlac
mock, 4
T2G, 4
0-2
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
20-22
22-24
1 2 3 4 5 6 7 8 9 10 11 12 13 14

C. MT2: BGMK PULSED AT 2 HR


Mock-0
T2G-0
Myx-cellular
secreted
0 0.5 1 2 4
0.5 1 3
1 2 3 4 5 6 7 8 9 10 11

D. MT2: BGMK PULSED AT 4 HR

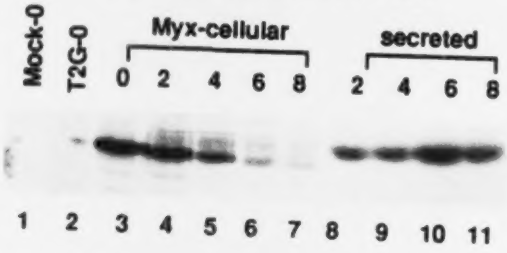

Mock-0
T2G-0
Myx-cellular
secreted
0 2 4 6 8
2 4 6 8
1 2 3 4 5 6 7 8 9 10 11

Figure VI-2. Endo H Sensitivity of Intracellular and Secreted M-T2 and Secreted M-T7 Proteins Synthesized in Infected BGMK and RL-5 Cells

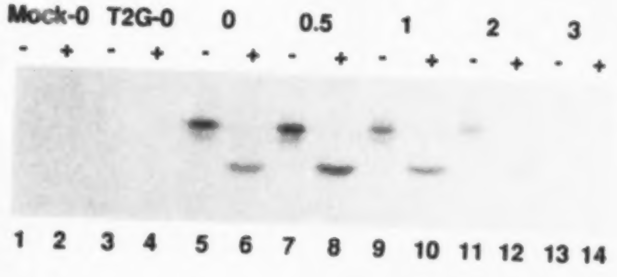
A. Endo H sensitivity of pulse-labelled M-T2 protein synthesized in infected BGMK cells and chased for varying amounts of time. At 2 hours post-infection BGMK were pulsed with [^{35}S] met/cys for 30 minutes, and labelled protein chased with cold medium for varying amounts of time, and M-T2 protein was immunoprecipitated from supernatants and lysates, and digested with Endo H. Immunoprecipitates from lysates of BGMK cells mock-, vMyxt2g- or vMyxlac immediately harvested (lanes 1-6) or chased with cold medium for 30 min, 1, 2, and 3 hours (lanes 7-14), undigested (lanes 1, 3, 5, 7, 9, 11 and 13) or digested with Endo H (lanes 2, 4, 6, 8, 10, 12 and 14).

B. Endo H sensitivity of pulse-labelled M-T2 protein synthesized in infected RL-5 cells and chased for varying amounts of time. At 2 hours post-infection RL-5 were pulsed with [^{35}S] met/cys for 30 minutes, and labelled protein chased with cold medium for varying amounts of time, M-T2 protein was then immunoprecipitated from supernatants and lysates, and digested with Endo H. Immunoprecipitates from lysates of RL-5 cells mock-, vMyxt2g-, or vMyxlac immediately harvested (lanes 1-6) or chased with cold medium for 1, 2, 3, 4 and 5 hours (lanes 7-16), undigested (lanes 1, 3, 5, 7, 9, 11, 13, 15) or digested with Endo H (lanes 2, 4, 6, 8, 10, 12, 14, 16).

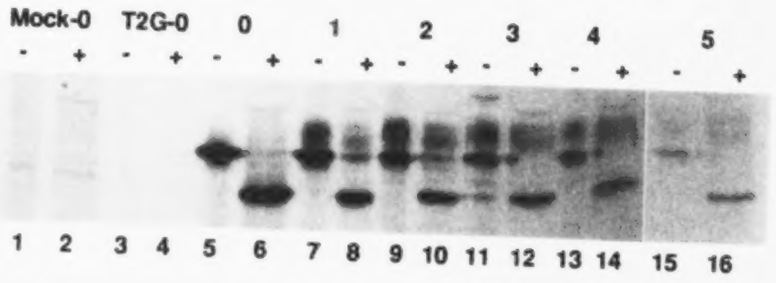
C. Endo H sensitivity of intracellular and secreted M-T2 protein synthesized from myxoma-infected RL-5 cells. Infected RL-5 cells were cultured in the presence of [^{35}S] met/cys for 16 hours, after which the M-T2 protein present in cell lysates and supernatants was immunoprecipitated. Immunoprecipitates from lysates from vMyxt2g- (lanes 1,2) and vMyxlac-infected cells (lanes 3,4) and supernatants from vMyxt2g- (lanes 5, 6) and vMyxlac-infected cells (7, 8), untreated (lanes 1, 3, 5 and 7) and treated (lanes 2, 4, 6 and 8) with Endo H.

D. Endo H sensitivity of M-T2 secreted from myxoma infected BGMK cells. M-T2 immunoprecipitates from supernatants from BGMK cells infected in the presence of [^{35}S] met/cys and harvested at 4 hours post-infection, mock-(lanes 1,2), vMyxt2g- (lanes 3, 4) and vMyxlac-infected (lanes 5, 6), untreated (lanes 1, 3 and 5) and treated (lanes 2, 4 and 6) with Endo H.

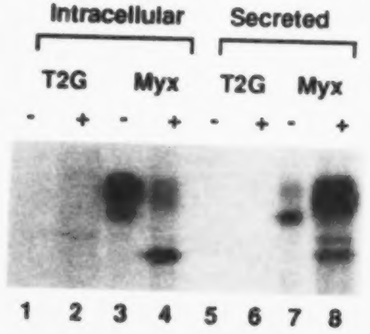
E. Endo H sensitivity of M-T7 produced from infected BGMK and RL-5 cells. BGMK and RL-5 cells, mock- or vMyxlac infected, were labelled 4 hours prior to harvest, after which secreted M-T7 protein was immunoprecipitated. M-T7 immunoprecipitates from supernatants from BGMK cells mock- (lanes 1,2) or vMyxlac-infected (lanes 3, 4) harvested 4 hours post-infection, or vMyxlac-infected harvested 8 hours post-infection (lanes 5, 6) or from RL-5 cells mock- (lanes 7, 8) or vMyxlac-infected (lanes 9, 10) harvested 4 hours post-infection, or vMyxlac-infected harvested 8 hours post-infection (lanes 11, 12), untreated (lanes 1, 3, 5, 7, 9 and 11) and treated (lanes 2, 4, 6, 8, 10, 12) with Endo H.


A. M-T2: BGMK INTRACELLULAR

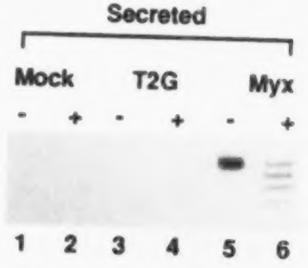
Mock-0 T2G-0 0 0.5 1 2 3
- + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14


B. M-T2: RL-5 INTRACELLULAR

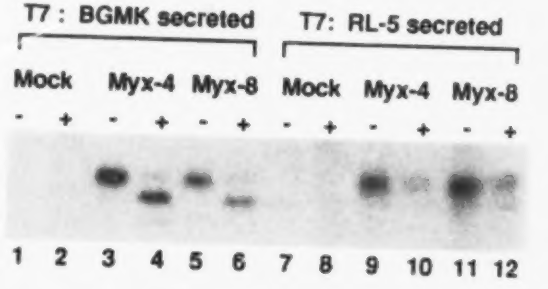
Mock-0 T2G-0 0 1 2 3 4 5
- + - + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


C. M-T2: RL-5

Intracellular
Secreted
T2G Myx T2G Myx
- + - + - + - +
1 2 3 4 5 6 7 8


D. M-T2: BGMK

Secreted
Mock T2G Myx
- + - + - +
1 2 3 4 5 6


E. M-T7: BGMK AND RL-5 SECRETED

T7 : BGMK secreted
T7: RL-5 secreted
Mock Myx-4 Myx-8 Mock Myx-4 Myx-8
- + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12

Figure VI-3. Northern Analysis of M-T2 Message in Infected BGMK Cells

A. BGMK cells were mock-, vMyxt2g, or vMyxlac-infected in the absence or presence of araC for varying amounts of time, after which RNA was prepared and analyzed by Northern blotting, probing with a 0.9 kb radiolabelled M-T2 probed as outlined under Materials and Methods. RNA prepared from cells mock-infected (lane 1), vMyxt2g- (lane 2) and vMyxlac-infected (lane 3) 2 hours, vMyxlac-infected 12 hours in the presence and absence of arabinosyl cytosine (lanes 4 and 5), vMyxt2g-infected 12 hours (lanes 6) is shown. Size standards (6.3, 2.4, 1.6, 1.0 and 0.6 kb) are indicated.

B. RNA prepared from vMyxlac-infected BGMK cells harvested at 3, 6, 9, 12, 18 and 24 hours post infection (lanes 1-6) is shown. Ribosomal RNA migrating at 6.3 and 2.4 kD are indicated.

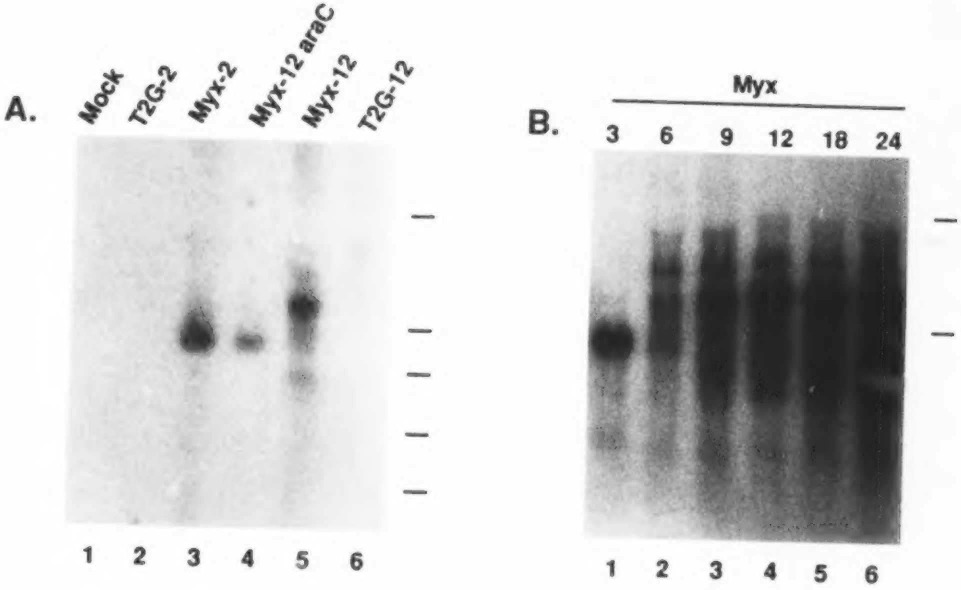

A.
Mock
T2G-2
Myx-2
Myx-12 araC
Myx-12
T2G-12
1
2
3
4
5
6
B.
Myx
3
6
9
12
18
24
1
2
3
4
5
6

Figure VI-4 Growth of RL-5 cells in the Presence and Absence of Rabbit TNF

RL-5 cells were cultured in the presence (triangles) and absence (boxes) of 50 nM purified rabbit TNF, and the number of cells present counted up to 5 days of culturing, as outlined under Materials and Methods. Standard deviations of triplicate determinations are shown.

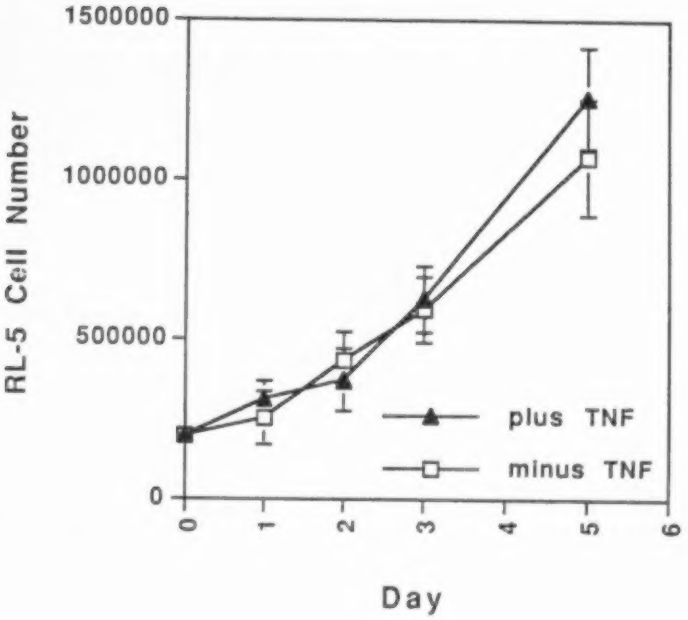

1500000
1000000
500000
0
RL-5 Cell Number
0
1
2
3
4
5
6
Day
plus TNF
minus TNF

Figure VI-5. Cytotoxicity of RL-5 Cells to L929-8 Cells

Actinomycin-D-sensitized L929-8 cells were overlayed with increasing numbers of RL-5 cells alone or in the presence of 2.5 µg/ml anti rabbit TNF antibody or 100 nM purified M-T2 protein, or with increasing numbers vMyxlac-infected or vMyxt2g-infected RL-5 cells for 18 hours, after which L929-8 viability was determined by neutral red uptake as outlined under Materials and Methods. Standard deviations of quadruplicate determinations are shown. Inset shows the ability of the anti RTNF antibody to inhibit the cytolytic effect of rabbit TNF. Actinomycin-D-sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF alone or together with 2.5 µg/ml anti rabbit TNF antibody, and cell viability determined by neutral red uptake after 18 hours.

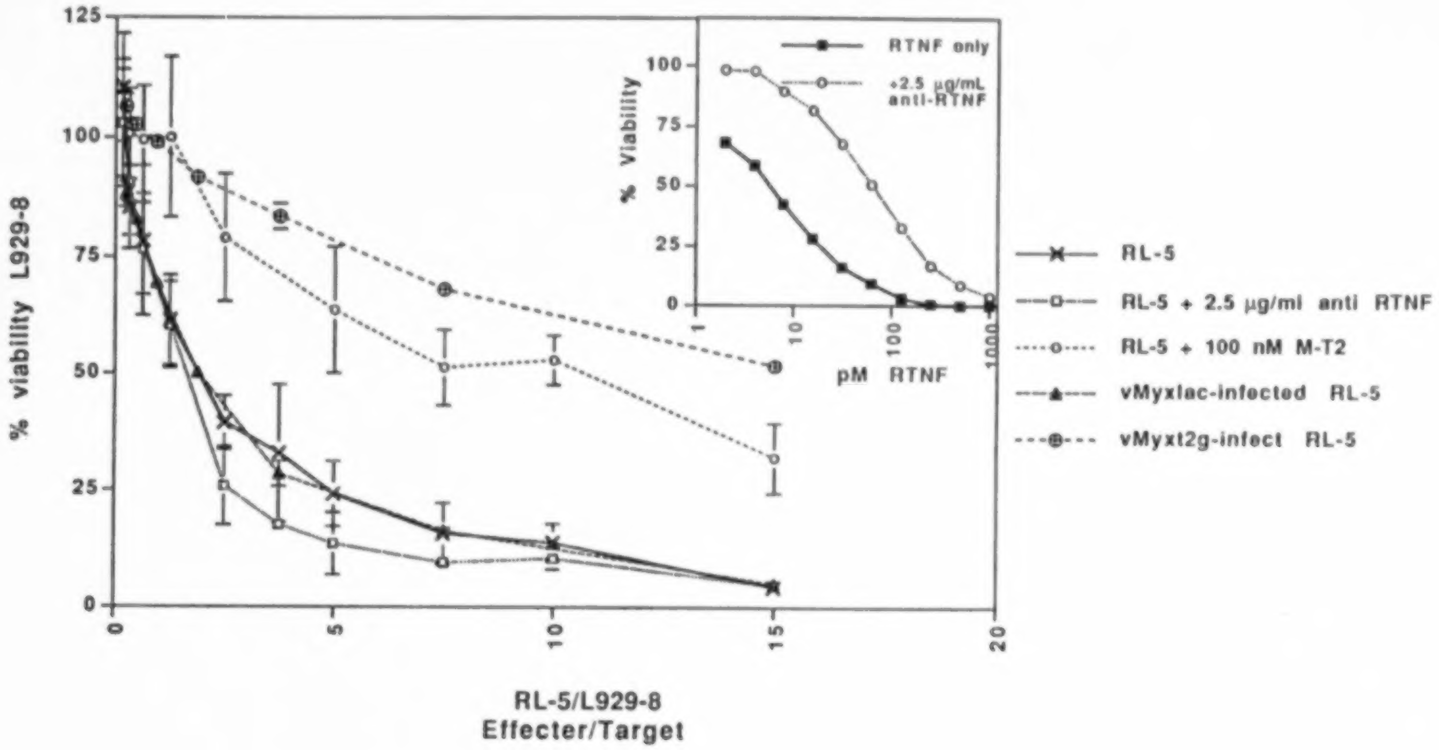

% viability L929-8
125
100
75
50
25
0
0
5
10
15
20
RL-5/L929-8
Effecter/Target
% Viability
100
75
50
25
0
1
10
100
1000
pM RTNF
RTNF only
+2.5 µg/mL anti-RTNF
RL-5
RL-5 + 2.5 µg/ml anti RTNF
RL-5 + 100 nM M-T2
vMyxlac-infected RL-5
vMyxt2g-infect RL-5

Figure VI-6. Structure of the Different Myxoma Viruses in which the M-T2 Genes have been Inactivated, Reconstituted or Truncated

A. Strucure of 160 kDa myxoma virus showing the location of the M-T2 genes within each copy of the terminal inverted repeats (TIRS) in wild type myxoma, vMyx(wt), vMyxlac, and the M-T2 reconstitution virus, vMyxt2r, the insertion of a gpt cassette into the M-T2 gene in vMyxt2g, and the insertion of a β-galactosidase cassette within the Bam E fragment in vMyxlac and vMyxt2g. Heavy arrows designate the TIRs, while light arrows designate open reading frames.

B. Structure of the different myxoma virus M-T2 truncations with boxes terminating at the point of each truncation and full length M-T2 with lines indicating cysteine residues, shading indicating the cysteine rich repeats, asterisks designating N-glycosylation sites, and a vertical arrow designating the secretion leader cleavage site, and a bent arrow showing the vaccinia synthetic late promoter that controls M-T2 expression.

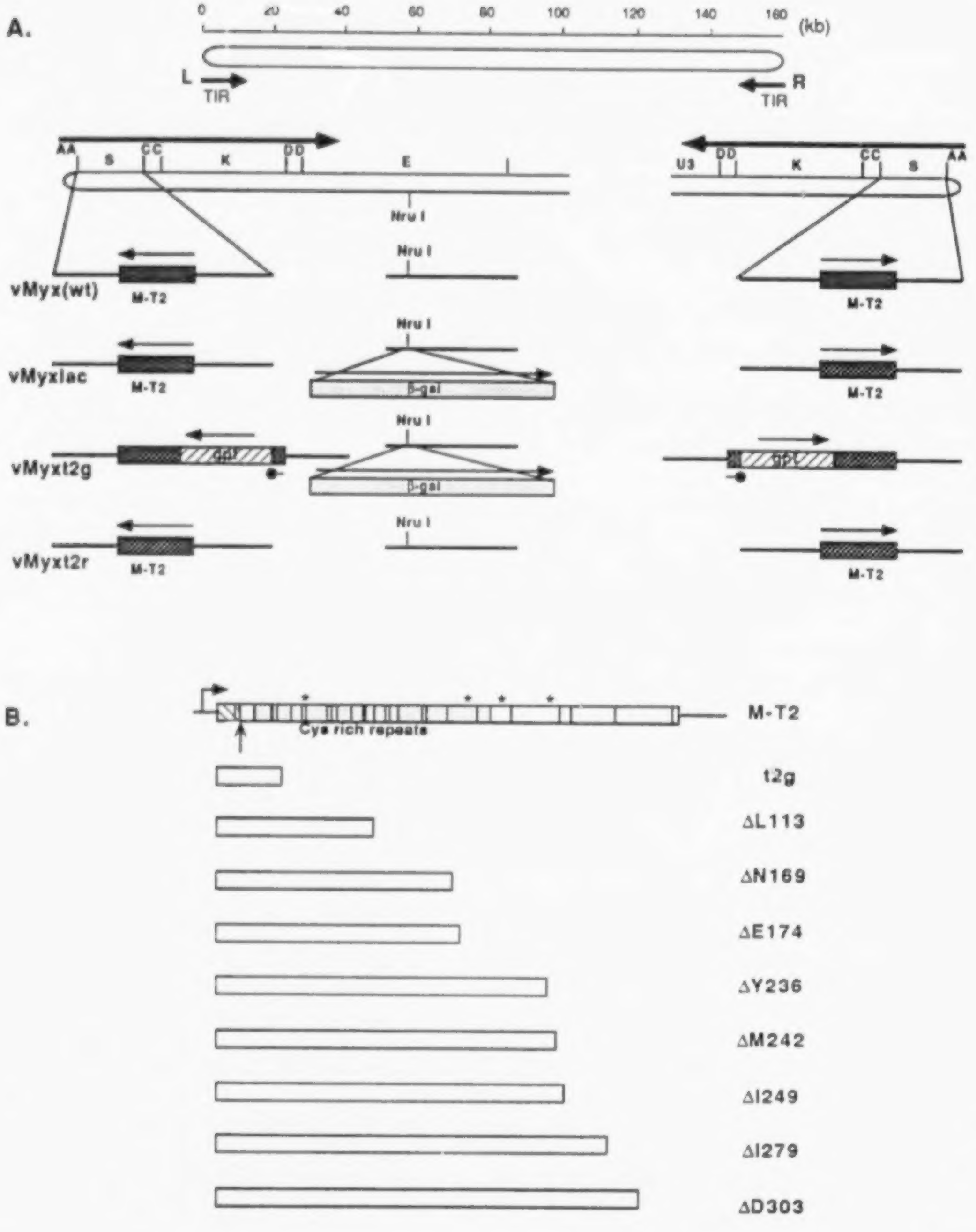

A.
0
20
40
60
80
100
120
140
160
(kb)
L
TIR
R
TIR
AA
S
CC
K
DD
E
U3
DD
K
CC
S
AA
Nru I
vMyx(wt)
M-T2
M-T2
vMyxlac
M-T2
β-gal
M-T2
vMyxt2g
gpt
β-gal
gpt
vMyxt2r
M-T2
M-T2
B.
M-T2
Cys rich repeats
t2g
ΔL113
ΔN169
ΔE174
ΔY236
ΔM242
ΔI249
ΔI279
ΔD303

Figure VI-7. Coimmunoprecipitation of Rabbit TNF with Full Length M-T2 and the M-T2 Truncations

[^{35}S]-rabbit TNF, present in supernatants from VV-T2RaTNF infected cells was immunoprecipitated with an anti-rabbit TNF antibody (lane 1) or combined with lysates from cells infected with vMyxlac (lane 2), vMyxt2g (lane 11) or myxoma viruses expressing the M-T2 C-terminal truncations ΔL113, ΔN169, ΔE174, ΔY236, ΔM242, ΔI249, ΔI279 and ΔD303 (lanes 3-10), immunoprecipated with an anti-MT2 antibody as described under the Materials and Methods.

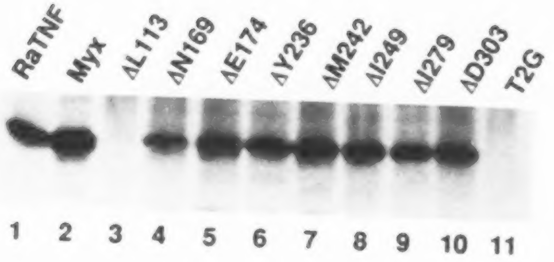
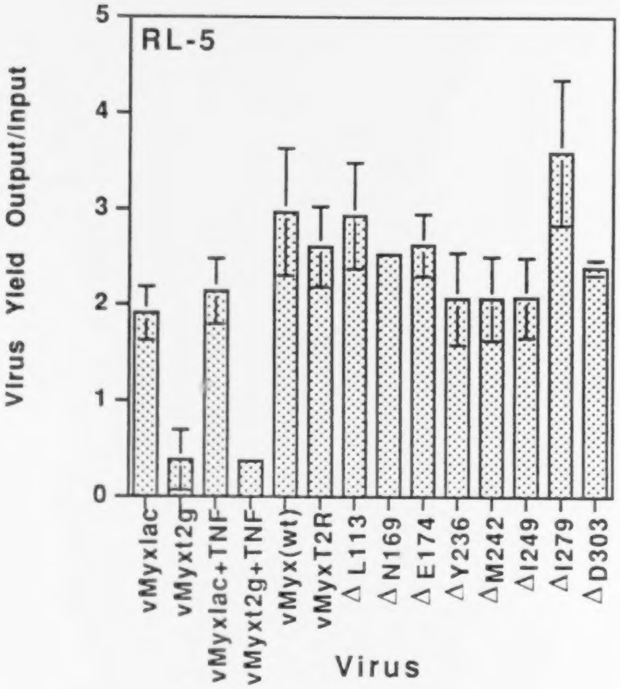

RaTNF
Myx
ΔL113
ΔN169
ΔE174
ΔY236
ΔM242
ΔI249
ΔI279
ΔD303
T2G
1
2
3
4
5
6
7
8
9
10
11

Figure VI-8. Growth of the Different Myxoma Constructs Expressing Full-Length M-T2 and M-T2 Truncations in RL-5 and BGMK Cells

RL-5 (A) and BGMK (B) cells were infected at a m.o.i. of 1, and after 36, cells were harvested and virus titers determined by plaque assay as outlined under the Materials and Methods. Data is expressed as the virus yield, output p.f.u./input p.f.u. Error bars designate standard deviations of triplicate determinations. vMyxlac- and vMyxt2g-infected RL-5 cells were also continuously cultured in the presence of 50 nM rabbit TNF.

A.


RL-5
Virus Yield Output/Input
0
1
2
3
4
5
vMyxlac
vMyxt2g
vMyxlac+TNF
vMyxt2g+TNF
vMyx(wt)
vMyxT2R
Δ L113
Δ N169
Δ E174
Δ Y236
Δ M242
Δ I249
Δ I279
Δ D303
Virus

B.

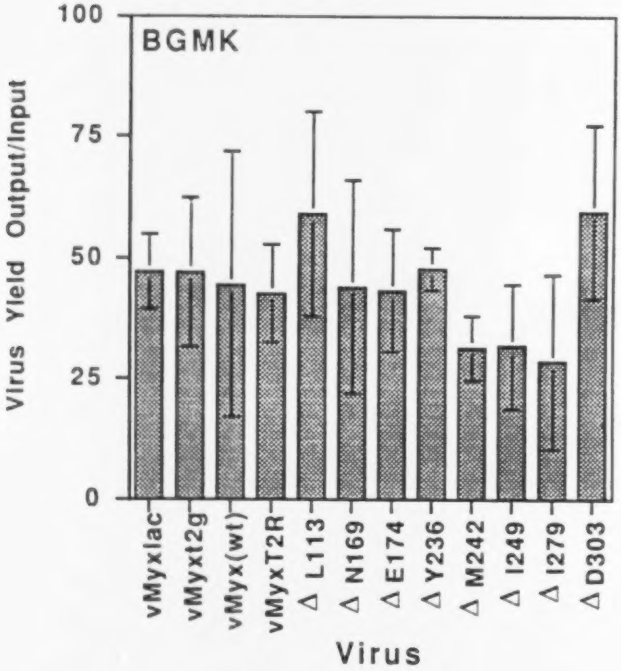

BGMK
Virus Yield Output/Input
0
25
50
75
100
vMyxlac
vMyxt2g
vMyx(wt)
vMyxT2R
Δ L113
Δ N169
Δ E174
Δ Y236
Δ M242
Δ I249
Δ I279
Δ D303
Virus

Figure VI-9. Quantitation of Apoptosis within Infected RL-5 Cells by FITC-12-dUPT Incorporatin and Flow Cytometric Analysis

RL-5 cells were infected with virus, 10 hours, after which fragmented DNA was stained with FITC-12-dUPT and the amount of fluorescence quantitated by flow cytometric analysis as described under the Materials and Methods. The relative cell number versus the fluorescence intensity of (A) mock- (B) vMyxlac-infected cells and overlays of vMyxlac-infected (dark) and (C) vMyxt2g (D) vMyxt2g-, infected in the presence of 500 nM M-T2 protein (E) vMyxMT2ΔL113- (F) vMyxMT2ΔN169- (G) vMyxMT2ΔDE174- (H) vMyxMT2ΔDY236 (I) vMyxMT2ΔM242 (J) vMyxMT2ΔI249 (K) vMyxMT2ΔI279 and (L) vMyxMT2Δd303-infected cells (outlines) are shown. (M) Culturing M-T2 with the vMyxt2g-infected RL-5 cells above did not affect its ability to inhibit rabbit TNF. The % viability of actinomycin D-sensitized L929-8 cells with increasing TNF concentration is shown for TNF only (squares), or in the presence of 5% RPMI media containing 500 nM M-T2 protein before (circles) or after (triangles) culturing vMyxt2g-infected RL-5 cells, as described under the Materials and Methods.

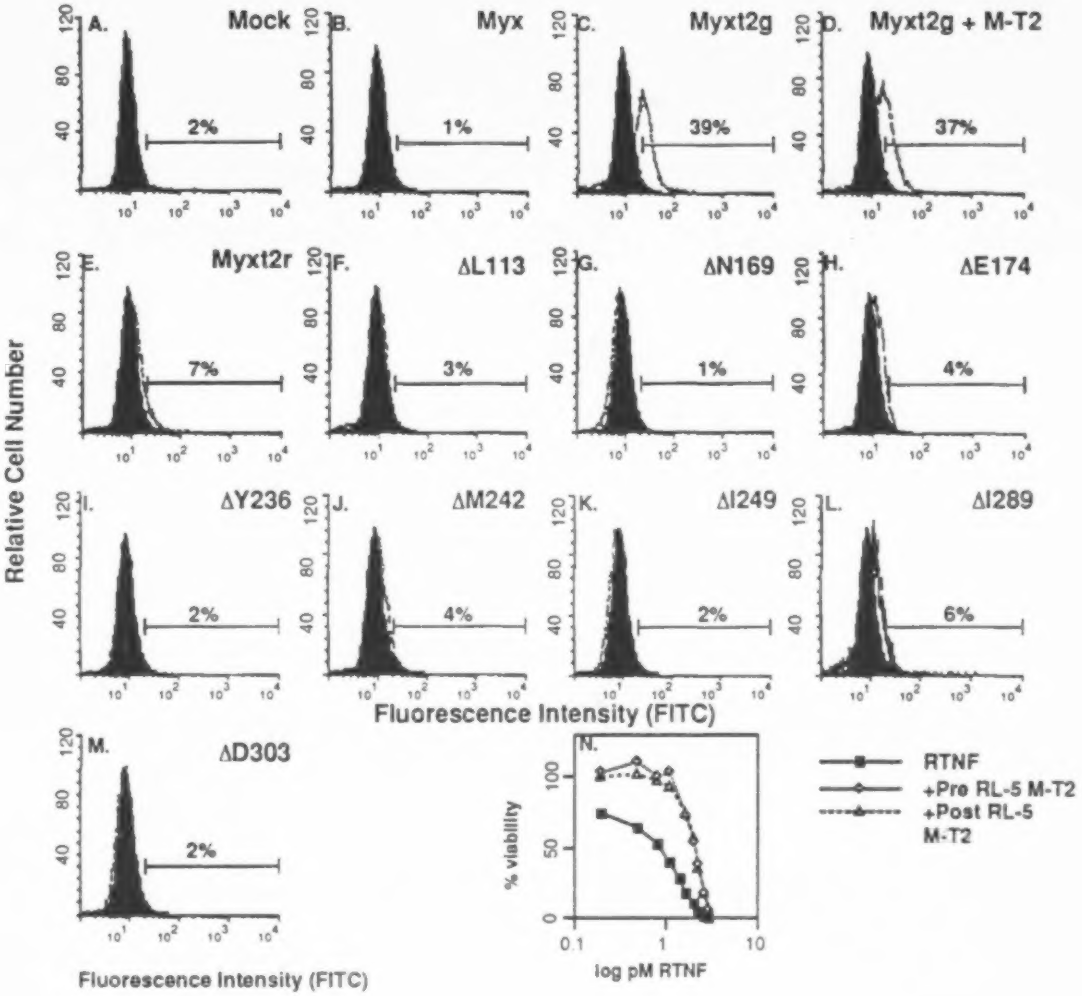

A. Mock 2%
B. Myx 1%
C. Myxt2g 39%
D. Myxt2g + M-T2 37%
E. Myxt2r 7%
F. ΔL113 3%
G. ΔN169 1%
H. ΔE174 4%
I. ΔY236 2%
J. ΔM242 4%
K. ΔI249 2%
L. ΔI289 6%
M. ΔD303 2%
Relative Cell Number
120
80
40
10^1
10^2
10^3
10^4
Fluorescence Intensity (FITC)
Fluorescence Intensity (FITC)
N.
% viability
100
50
0
0.1
1
10
log pM RTNF
RTNF
+Pre RL-5 M-T2
+Post RL-5 M-T2

Table VI-1. Soluble TNF Secreted from Rabbit Cells

Rabbit cells 2 x 10^6cells/ml	concentration secreted TNF
RL-5	<50 pg/ml
RL-5 (VV-601-infected)	<50 pg/ml
RL-5 + 1 µg/ml LPS	<50 pg/ml
RL-5 (vMyxlac-infected)	<50 pg/ml
RL-5 (vMyxt2g-infected)	<50 pg/ml
rabbit monocytes + 1 µg/ml LPS	200 ng/ml

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CHAPTER VII - SUMMARY AND FUTURE DIRECTIONS

Investigation of the strategies viruses use to evade host immune responses has led to the identification of proteins that specifically interfere with TNF signaling cascades, including the adenovirus proteins which inhibit TNF-mediated cytolysis and the cowpox crmA protein which inhibits both TNF- and Fas-induced apoptosis (1-3). The work presented in this thesis has characterized a different mechanism of TNF-inhibition. The secreted myxoma T2 protein sequesters the TNF ligand to prevent its association with its cellular receptors, and thus by inhibiting receptor clustering in this fashion, M-T2 short-circuits all subsequent TNF responses. Evidence for this mechanism of TNF inhibition include (a) the determination that the affinities between monomeric and dimeric M-T2 with rabbit TNF, which compare favourably to the affinities of TNF with cellular TNFRs (b) the demonstration that M-T2 protein was able to block the specific binding of rabbit TNF to the surface of rabbit cells and (c) the characterization of the ability of M-T2 to inhibit the cytolytic activities of rabbit TNF in which the molar amounts of dimeric, but not monomeric, M-T2 required for complete inhibition of TNF cytolytic activities are similar to the molar amounts of soluble TNFRs required to inhibit TNF. Figure VII-1 shows a model for M-T2 inhibition of rabbit TNF. Both shed forms of the receptor and monomeric M-T2 bind the trimeric TNF ligand, but two molecules of each must bind to prevent TNF from inducing dimerization of receptors. Binding of dimeric M-T2 protein results in a TNF complex incapable of signaling.

The species-specificity of both M-T2 protein and rabbit cells for rabbit TNF was also characterized during the course of this thesis. M-T2 protein inhibits only rabbit TNF, and rabbit cells, unlike mouse and human cells, were only sensitive to cytolysis by homologous rabbit TNF. As the species specificity and preference of more virulence factors encoded by viruses become clarified, the reasons why viruses cause disease pathologies within different host species will be better understood. However, it is still not known why the pathology of myxoma within *Oryctolagus* and *Sylvilagus* rabbits is dramatically different, and an understanding will require further knowledge on both myxoma-encoded virulence determinants and the significance of genetic differences between rabbit species which contribute to immune responses.

Athough SFV and myxoma share a close evolutionary relationship, they induce very different symptoms within *Oryctolagus*, and the reasons for this difference are not well understood. Both SFV and myxoma encode TNFR homologs, but they vary in species specificity. S-T2 protein is thought to bind and inhibit mouse and human TNF (K_d of 1 nM for mouse TNF, which compares with the M-T2 K_d reported here of 1.7 nM) (4), but it has not been determined if S-T2 has an anti-apoptotic effect within lymphocytes. It will be interesting to see whether S-T2 also inhibits the second M-T2 ligand target, expressed on RL-5 cell surfaces, but this experiment will require greater amounts of S-T2 protein than that present in supernatants from infected cells. SFV is unable to replicate efficiently within rabbit splenocytes or RL-5 cells (5, 6), and the inability of SFV to replicate within lymphocytes is thought to contribute to its inability to disseminate within rabbits to secondary sites, rendering it less virulent than myxoma (7, 8). Infection of RL-5 cells with SFV results in apoptosis despite the presence of S-T2, although for myxoma virus, M-T2 expression alone is not sufficient to inhibit virus infection-induced apoptosis of RL-5 cells (5), and the SFV genome might lack another apoptotic inhibitor encoded within the myxoma genome. A recombinant myxoma virus can be constructed in which *M-T2* sequence is replaced with *S-T2* sequence, to determine if M-T2 and S-T2 function analogously to inhibit apoptosis in RL-5 cells. Future experiments on host range and the ability of the two viruses to replicate within lymphoid subpopulations will contribute to our knowledge of the mechanisms of virus dissemination within the rabbit host and its significance to the development of myxomatosis.

Does TNF have a direct anti-viral effect on poxviral growth? Since TNF has been shown to directly kill certain virus-infected cells (1, 9), we tested to see if TNF inhibited poxvirus replication. Rabbit TNF had only a modest effect on the replication of myxoma and vaccinia (strain WR) viruses within TNF-sensitive rabbit RK-13 cells, and had no effect on myxoma yields from infected RL-5 cells (TNF insensitive). However, vaccinia (strain WR) encodes a homolog of the cowpox *crmA* gene, whose gene product has been shown to inhibit both TNF- and Fas-mediated apoptosis (3). In our experiments with vaccinia virus, the B13R protein was most likely inhibiting the TNF cytolytic signal and allowing for productive viral infection. It would be informative to see what effect TNF has on the replication of a *B13R* knockout vaccinia virus. Moreover, it is highly plausible that myxoma virus encodes a *crmA* homolog or another protein capable of inhibiting apoptosis. Thus, myxoma viruses in which either the M11L gene (apoptosis inhibitor) or SERP-1 gene (serine proteinase inhibitor) had been inactivated were tested to see what effect TNF had on their replication in tissue culture. As more myxoma virus virulence factors are studied, it will be useful to test the effects of TNF on the replication of the corresponding myxoma virus "gene knockouts", as a simple screen to check if these virulence determinants inhibit intracellular TNF-apoptotic pathways. Poxviruses may well ressemble adenoviruses which encode multiple factors that interfere with TNF cytolysis (1, 2).

Detailed kinetic studies of M-T2 synthesis and secretion have been reported here. M-T2 protein is synthesized at both early and late times of myxoma infection, but is only secreted at early times. Moreover, mRNA transcripts were observed hybridizing to an M-T2 genomic probe, although the sizes of the mRNA species hybridizing were different at early and late times. A thorough characterization of the 5' ends of these transcripts, by S1-analysis or primer extension, must be performed before we can say for sure that the mRNA species detected at late times are, in fact, transcripts capable of expressing M-T2 protein. Moreover, a slight decrease in M-T2 protein synthesis was observed between 6-

10 hours post-infection. Several vaccinia virus early promoters have been shown to be reactivated at late times of infection, with decreases in mRNA levels observed during the transition from early to late stage of infection (10). A careful examination of M-T2 transcript 5' initiation start sites during infection will tell whether the M-T2 promoter is such an early/late promoter. Furthermore, there is an unusual array of nine related tandem repeats immediately upstream of the M-T2 promoter which may effect M-T2 transcription (11). The significance of these sequences to M-T2 transcription could be determined by placing a reporter gene under the regulation of the M-T2 promoter, with and without the tandem repeats, and looking at expression throughout infection.

It was surprising that all the M-T2 C-terminal truncations expressed from both myxoma and vaccinia were preferentially retained within infected cells, which suggests that the C-terminal 24 amino acids of M-T2 are required for efficient secretion. To test this hypothesis, recombinant vaccinia viruses can now be constructed expressing the various M-T2 C-terminal truncations with the last 24 amino acids of full-length M-T2 added in frame to the C-terminus. If the resulting proteins are efficiently secreted, it would appear that the C-terminus of M-T2 facilitates efficient secretion from infected cells. Moreover, examination of the kinetics of M-T2 secretion revealed that M-T2 was retained within the cell at late times, although the reason for this retention is unknown. It is possible that M-T2 interaction with a cellular or viral protein leads to its retention and this might be the actual role of the C-terminal sequence of M-T2. Strategies to detect other M-T2 binding partners are discussed further on in the text. Alternatively, retention within the endoplasmic reticulum could be due to association of misfolded M-T2 truncation protein with calnexin (12). Antibodies against both M-T2 protein and calnexin are available, and co-immunoprecipitation experiments can be performed to determine if M-T2 retention is due to association with calnexin.

Intracellular M-T2 glycoprotein remains sensitive to digestion by Endo H, indicating that it is most likely present within the cell in a pre-medial Golgi compartment, such as the endoplasmic reticulum or cis Golgi. Knowledge of the exact intracellular location of M-T2 protein will be very useful in developing models for intracellular M-T2 inhibition of apoptosis. Preliminary immunofluorescence studies with myxoma-infected cells, probing with affinity-purified anti-M-T2 antibody, were hampered by high levels of background antibody binding and low levels of M-T2 expression within infected cells. It may be easier to localize M-T2 protein within VV-MT2-infected cells rather than myxoma-infected cells for fibroblast and epithelial cell lines such as BGMK and RK-13; however, vaccinia virus, itself, induces apoptosis within RL-5 cells, and for these cells, myxoma virus must be used. Moreover, the exact localization of M-T2 within subcellular compartments may require techniques such as confocal microscopy.

Several approaches can be used to identify the cytotoxic ligand that is expressed on the surface of RL-5 cells and that is inhibitable by M-T2. Based on the homology between M-T2 and other members of the TNFR superfamily, likely candidates include apoptosis-associated members of the TNF superfamily. Thus, RL-5 cells could be tested with available antibodies recognizing ligands within the TNF superfamily, which may reveal whether or not RL-5 cells express known cytolytic ligands such as Fas, CD40L or LTα/β on their cell surfaces. Antibodies which cross-react with RL-5 cell surface proteins can then be tested to determine whether they are also capable of inhibiting the cytolysis of L929-8 cells by RL-5 cells. Negative results will be difficult to interpret, though, because one would be unsure if RL-5 cells do not express that ligand or if the antibody directed against the mouse or human ligand is species specific and does not recognize the rabbit ligand expressed on RL-5 cells. Alternatively, to determine what factors L929-8 cells are sensitive to in addition to TNF, L929-8 cells can be treated with agonist antibodies to the receptors for various ligands, or alternatively with membrane preparations containing the ligand, to see if they are cytolytic for L929-8 cells. Ruby *et al* (13) report that pretreating HSV-1-infected L929 cells with membrane preparations containing CD40L results in a decrease in virus yield comparable with the decrease observed when cells were pretreated with mouse TNF, indicating that L929 cells (and probably L929-8 cells as well) do show sensitivity to CD40L. It would be interesting to see if membrane preparations of CD40L are cytolytic to actinomycin-D sensitized L929-8 cells, and if they are, test whether M-T2 protein is able to inhibit cytolysis. Moreover, similar membrane preparations expressing FasL can also be tested to see if they induce cytolysis. However, once again, if M-T2 is unable to inhibit the mouse or human forms of these ligands, one is still unsure if such a result is due to species specificity or an inability of M-T2 to bind even the corresponding rabbit ligand. However, if antibodies and membrane preparations containing ligands are available, the above experiments are simple and quick to perform and do not require a major investment of time.

Does M-T2 association with another viral or cellular protein lead to its retention at late times during infection? Does inhibition of apoptosis within RL-5 cells require the association of M-T2 with another protein? In an attempt to observe proteins associating with M-T2, cell lysates were prepared from [^{35}S]met/cys-labelled RL-5 cells, infected with myxoma virus, and immunoprecipitations were performed with anti-MT2 antibody. But the results were difficult to interpret because there was a problem with high background under mild wash conditions. However, whenever antibodies directed against TNF family/TNFR family members or other apoptotic determinants are available that cross-react with the rabbit protein, immunoprecipitations should be performed from myxoma RL-5 cell lysates using anti-MT2 antibody, and probing Western blots of the immune complexes with the antibody directed against the potential TNF binding partner. Moreover, such a strategy can also be used to screen for another myxoma-encoded protein which may associate with M-T2, using available antibodies against myxoma proteins.

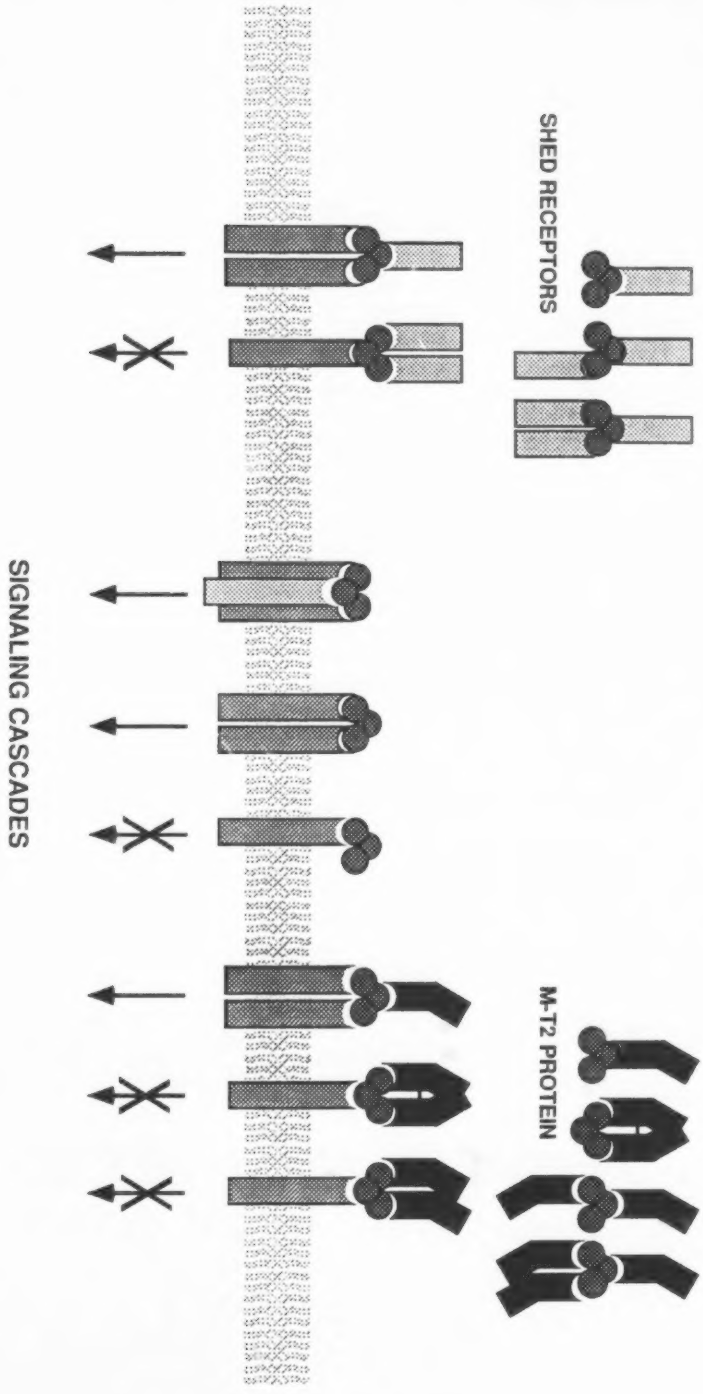
In the last few years, a plethora of binding proteins have been identified for various factors by yeast two-hybrid selection (14), and two-hybrid screening may be the most promising strategy to identify any cellular factors which M-T2 associates with, especially if these other factors are not members of the TNF/TNFR superfamilies. DNA containing M-T2, M-T2 (residues 1-113, the apoptosis inhibition domain), M-T2 (residues 27-194, the four CRDs forming the TNF-binding domain) and M-T2 (residues 303-326, C-terminal sequences which may be important for efficient secretion) can be cloned in frame into a yeast GAL4 DNA-binding domain vector, and the resulting plasmids used as bait in two-hybrid screens of GAL4-activation domain cDNA libraries. To optimize chances of success, rabbit cDNA libraries, such as a rabbit splenocyte or RL-5 library should be used, although this will entail first constructing the GAL-4 activation domain cDNA library. Lisa Sedger in the McFadden lab is presently constructing such a library. Moreover, a myxoma GAL-4 activation domain cDNA library will be useful to screen for interactions between myxoma virus proteins.

Finally, the recombinant myxoma virus, vMyx-MT2ΔL113 may be very useful to determine, *in vivo*, the contributions M-T2 inhibition of TNF makes to myxoma virus evasion of rabbit immune responses. ΔL113 does not bind or inhibit rabbit TNF, but it is able to inhibit viral infection induced apoptosis in infected $CD4^+$ T cells (RL-5). If the phenotype of vMyx-MT2ΔL113 in European rabbits ressembles its parental virus, vMyxlac, in that full-blown myxomatotis is observed, it would appear that M-T2 inhibition of TNF does not contribute significantly to pathogenesis *in vivo*, but rather it is M-T2 inhibition of apoptosis which contributes to the severity of the illness. But if the phenotype of vMyx-MT2ΔL113 ressembles vMyxt2g, and the majority of rabbits recover infection, it would indicate that an ability to inhibit TNF contributes to the virulence of myxoma virus in European rabbits. Note however, that some poxviruses (e.g. cowpox) actually encode multiple secreted proteins that bind and inhibit TNF, so the search for other myxoma proteins that might contribute to TNF modulation remains a worthwhile goal.

Thus, work presented in this thesis has contributed to our understanding of how the M-T2 gene product of myxoma virus contributes to viral virulence, and has shown that TNF is a relevant target for M-T2 action. In the course of these studies, more questions have arisen on the mechanisms by which M-T2 inhibits host immune responses, and the answers to these questions await future experimentation.

Figure VII-1. A Model of M-T2 Inhibition of Rabbit TNF

Clustering of membrane TNF receptors (▨), either dimerization or trimerization, by trimeric TNF ligand (●) leads to signal transduction cascades. Both M-T2 protein (■) and shed forms of the receptors are able to bind TNF ligand, and prevent its association with cellular receptors. Two M-T2 monomers or two shed receptors must bind the ligand to inhibit signaling, whereas binding of dimeric M-T2 results in a TNF/M-T2 complex incapable of triggering signaling cascades.


SIGNALING CASCADES
M-T2 PROTEIN
SHED RECEPTORS

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